

## REMARKS

### Summary of amendments

Claims 78-93 are pending. Claims 78 and 93 have been amended to recite B-cell antibodies. Claim 93 also has been amended to recite antibodies that have multiple epitope or multiple antigen specificity, based on the disclosure on page 13, lines 2-5. Claims 87-92 have been canceled. Claims 94-101 have been added. Claims 94-96 are supported by the disclosure at page 13, lines 19-23. Claims 97-101 are supported by the disclosure at page 8, line 27 to page 9, line 9. Claims 78-86 and 93-101 remain in the case. A new, more descriptive title has been provided.

Applicant respectfully requests that the foregoing amendments be made prior to examination of the present application, and respectfully requests reconsideration of the present application in view of the foregoing amendments and the reasons that follow. This amendment adds, changes and/or deletes claims in this application. A detailed listing of all claims that are, or were, in the application, irrespective of whether the claim(s) remain under examination in the application, is presented, along with appropriate defined status identifiers.

### Interview summary

Applicant would like to thank Examiners Gamble and Crowder for the courteous interview of May 17, 2007. The interview was most helpful in formulating claim language that would obviate the current rejection for lack of written description. Based on the comment at the interview that the amendment of the claims to recite "B-cell antibodies" instead of "antibody or antibody fragment specific to a marker associated with a B cell" would obviate the written description rejections, applicant has eliminated any further discussion of the merits of the outstanding Section 112, first paragraph rejection. Page 12, lines 12-16 describes "**antibodies and fragments against** bone marrow cells, particularly hematopoietic progenitor cells, pancreatic islet cells, spleen cells, parathyroid cells, uterine endometrium, ovary cells, testicular cells, thymus cells, **B-cells**..." Moreover, since this disclosure was present in the parent applications, USSN 09/110,181 and USSN 07/866,789, these provide a written description of the invention as presently claimed. Therefore applicant is entitled to a claim for domestic priority based on these earlier applications.

### B-cell antibodies were readily available circa 1992 and earlier

There were many B-cell antigens antibodies that were publicly available prior to the 1992 priority date of the present claims. Various murine B-cell antibodies were taught for treating NHL patients as early as the late 1980's. See, for example, Press *et al.*, "Monoclonal antibody 1F5 (anti-

CD20) serotherapy of human B cell lymphomas,” *Blood*, 1987 Feb;69(2):584-91; Press *et al.*, “Treatment of refractory non-Hodgkin's lymphoma with radiolabeled MB-1 (anti-CD37) antibody,” *J Clin Oncol.*, 1989 Aug;7(8):1027-38; and Nadler *et al.*, “Anti-B1 monoclonal antibody and complement treatment in autologous bone-marrow transplantation for relapsed B-cell non-Hodgkin's lymphoma,” *Lancet*, 1984 Aug 25;2(8400):427-31. Other B cell antibodies were known. For example, Mason *et al.* disclose anti-CD22 antibodies To15 and 4KB128, anti-CD19 antibody B4 and anti-CD21 antibody B2 (*Blood*, 1987 Mar;69(3):836-40). The Dana Farber group developed the B1 Mab against CD20 in the 80's and licensed it to Coulter for flow cytometry, and B-cell antibodies against other antigens, such as CD19, also were routinely used to identify B-lymphocytes by immunocytology of blood and tissue specimens. These antibodies were commercially available from various companies, including Becton-Dickinson and others. Moreover, Anderson *et al.* demonstrates that the making of antibodies to B cell antigens was within the level of skill in the art circa 1984, the date of his article (Anderson *et al.*, “Expression of human B cell-associated antigens on leukemias and lymphomas: a model of human B cell differentiation,” *Blood*, 1984 Jun;63(6):1424-33 - “The techniques of immunization, somatic cell hybridization, and selection of hybridomas that were used to produce the monoclonal antibodies directed at the Ia, B4, CALLA, Bi, B2, slg, Ti, Tb, PC- I , and PCA- 1 antigens have been previously described”). Copies or abstracts of the cited articles are appended to this response.

Applicant's contribution, as recited in the present claims, is the teaching that B-cell antibodies are useful in the treatment of immune diseases. Armed with this information, the skilled artisan could readily practice this teaching by using commercially available B-cell antibodies or by making other B-cell antibodies according to established methodologies, as supported by Anderson *et al.*

### **Rejection of claims 87-92 directed to LL2 antibody under Section 112**

Claims 87-92 were rejected under the first and second paragraphs of Section 112 based on their recitation of “LL2 antibody.” While not agreeing with the basis for this rejection, claims 87-92 have been canceled in order to advance prosecution. Withdrawal of this ground of rejection is therefore respectfully requested.

### **Rejections of claims 78-92 under Section 102**

Claims 78-92 are rejected under Section 102 based on various documents. WO93/19668 is the published PCT application corresponding to the parent of the present application. Since the amended claims clearly are entitled to their 1992 priority date, WO93/19668 is not a reference

against the present claims. Similarly, Hansen *et al.* (US Patent 5,443,953) was filed on December 8, 1993, after the April 7, 1992 filing date to which the present application claims domestic priority. Goldenberg *et al.* (US Patent 7,074,403) also was filed after the priority date of April 7, 1992 for the present application. Reconsideration and withdrawal of these rejections are respectfully requested.

### **Rejection of claims 78-92 for obviousness-type double patenting**

Claims 78-92 were rejected under the doctrine of non-statutory obviousness double-patenting based on the claims of US 6,653,104 and US 7,074,403. Forwarded herewith are terminal disclaimers that obviate these rejections.

### **Common ownership of US 7,074,403 and the invention claimed in the present application**

The examiner also calls upon applicant to resolve another issue with respect to US 7,074,403, by indicating the relationship between the present application and the issued patent. The present application and US 7,074,403 were, at the time the invention claimed in US 7,074,403 was made, owned by Immunomedics, Inc. Indeed, both inventions have been owned at all times since they were made by Immunomedics, Inc.

### **Conclusion**

In view of the foregoing, it is believed none of the references, taken singly or in combination, disclose the claimed invention, and therefore a notice of allowance is respectfully requested. If there are any problems with this response, or if the examiner believes that a telephone interview would advance the prosecution of the present application, Applicant's attorney would appreciate a telephone call.

Respectfully submitted,

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MAY 24, 2007

DATE

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***Press et al. "Treatment of refractory non-hodgkin's lymphoma with radiolabeled mb-1 (anti-cd37) antibody," J Clin Oncol., 1989 Aug;7(8):1027-38.***

The biodistribution, toxicity, and therapeutic potential of anti-cd37 monoclonal antibody (moab) mb-1 labeled with iodine 131 (<sup>131</sup>I) was evaluated in ten patients with advanced-, low- or intermediate-grade non-hodgkin's lymphomas who failed conventional treatment. Sequential dosimetric studies were performed with escalating amounts of antibody mb-1 (0.5, 2.5, 10 mg/kg) trace-labeled with 5 to 10 mCi <sup>131</sup>I. Serial tumor biopsies and gamma camera imaging showed that the 10 mg/kg moab dose yielded the best moab biodistribution in the ten patients studied. Biodistribution studies in the five patients with splenomegaly and tumor burdens greater than 1 kg indicated that not all tumor sites would receive more radiation than normal organs, and these patients were therefore not treated with high-dose radioimmunotherapy. The other five patients did not have splenomegaly and had tumor burdens less than 0.5 kg; all five patients in this group showed preferential localization and retention of moab at tumor sites. Four of these patients have been treated with <sup>131</sup>I (232 to 608 mCi) conjugated to anti-cd37 moab mb-1, delivering 850 to 4,260 Gy to tumor sites. Each of these four patients attained a complete tumor remission (lasting 4, 6, 11+, and 8+ months). A fifth patient, whose tumor did not express the cd37 antigen, was treated with <sup>131</sup>I-labeled anti-cd20 moab 1f5 and achieved a partial response. Myelosuppression occurred 3 to 5 weeks after treatment in all cases, but there were no other significant acute toxicities. Normal B cells were transiently depleted from the bloodstream, but immunoglobulin (Ig) levels were not affected, and no serious infections occurred. Two patients required reinfusion of previously stored autologous, purged bone marrow. Two patients developed asymptomatic hypothyroidism 1 year after therapy. The tolerable toxicity and encouraging efficacy warrant further dose escalation in this phase I trial.

PMID: 2666588 [pubmed - indexed for medline]

***Nadler et al., "Anti-B1 monoclonal antibody and complement treatment in autologous bone-marrow transplantation for relapsed B-cell non-Hodgkin's lymphoma," Lancet, 1984 Aug 25;2(8400):427-31.***

Eight patients with relapsed B-cell non-Hodgkin's lymphoma were treated with intensive chemoradiotherapy and reconstituted with autologous bone marrow rendered free of tumour cells by the B-cell-specific monoclonal antibody anti-B1 and complement. Before the autologous marrow transplantation patients were induced with chemotherapy, radiotherapy, or both, into a minimum disease state with less than 5% bone-marrow involvement with tumour. All patients treated achieved a complete clinical response and had stable haematological engraftment by 8 weeks. No significant acute or chronic toxic effects have occurred. B cells could be detected by 2 months after transplantation and normal immunoglobulin levels were achieved by 6 months. Six of eight patients are disease free in unmaintained remission more than 20, 19, 10, 8, 5, and 3 months after transplantation.

PMID: 6147502 [PubMed - indexed for MEDLINE]

# Monoclonal Antibody 1F5 (Anti-CD20) Serotherapy of Human B Cell Lymphomas

By Oliver W. Press, Frederick Appelbaum, Jeffrey A. Ledbetter, Paul J. Martin, Joyce Zarling, Pamela Kidd, and E. Donnell Thomas

Four patients with refractory malignant B cell lymphomas were treated with continuous intravenous (IV) infusions of murine monoclonal antibody (MoAb) 1F5 (anti-CD20) over five to ten days. Dose-dependent levels of free serum 1F5 were detected in all patients. Two patients had circulating tumor cells and in both cases 90% of malignant cells were eliminated from the blood stream within four hours of initiation of serotherapy. Antigenic modulation did not occur, and sustained reduction of circulating tumor cells was observed throughout the duration of the infusions. Serial bone marrow aspirations and lymph node biopsies were examined by immunoperoxidase and immunofluorescence techniques to ascertain MoAb penetration into extravascular sites. High doses (100 to 800 mg/m<sup>2</sup>/d and high serum 1F5 levels (13 to 190 µg/mL) were required to

coat tumor cells in these compartments in contrast to the low doses that were adequate for depletion of circulating cells. Clinical response appeared to correlate with dose of MoAb administered with progressive disease (52 mg), stable disease (104 mg), minor response (1,032 mg), and partial response (2,380 mg) observed in consecutive patients. The patient treated with the highest 1F5 dose achieved a 90% reduction in evaluable lymph node disease, but the duration of this remission was brief (six weeks). This study demonstrates that high doses of 1F5 can be administered to patients with negligible toxicity by continuous infusion and that clinical responses can be obtained in patients given >1 g of unmodified antibody over a ten-day period.

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**M**ONOCLONAL ANTIBODY (MoAb) serotherapy of malignancy represents a theoretically attractive, potentially nontoxic approach for the treatment of neoplastic disease.<sup>1-3</sup> Preliminary animal experimentation has demonstrated both the effectiveness and limitations of MoAbs that recognize tumor-associated antigens in preventing growth of murine hematologic malignancies.<sup>4,5</sup> Early human trials have shown that infusion of antibodies recognizing lymphoid cell differentiation antigens is a well-tolerated therapy capable of coating tumor cells and causing tumor regression in some patients.<sup>6-8</sup> However, the antitumor effectiveness of MoAbs has been limited by the presence of circulating free antigen, antigenic modulation, development of human antimouse antibodies (HAMA), emergence of antigen-negative tumor cell variants, and the inadequacy of host effector cell mechanisms.<sup>5,8-11</sup>

Here we present findings in four patients with B cell lymphomas treated with a murine IgG2a MoAb (1F5) chosen to avoid many of the previously encountered obstacles. MoAb 1F5 recognizes a 35,000 dalton antigen (Bp35,

CD20) present on the surface of normal and malignant B cells<sup>12</sup> that is not shed from the cell surface (unpublished observations), does not modulate in response to MoAb binding, and does not bind to any other normal tissues. Consequently, prolonged continuous MoAb 1F5 therapy can be administered without inducing the unresponsiveness to therapy that has necessitated intermittent bolus therapy in previous trials.<sup>8-11</sup> We have administered 1F5 by continuous intravenous (IV) infusion (52 to 2,380 mg over five to ten days) to determine toxicity, kinetics, penetration to extravascular tissues, and efficacy. Our studies have shown 1F5 to be a minimally toxic therapy capable of depleting circulating tumor cells at low doses and lymph node tumor cells at high doses. However, responses were transient, suggesting that antibodies conjugated to toxins or radioisotopes might afford more lasting clinical benefit than unmodified antibody.

## MATERIALS AND METHODS

**Antibody preparation.** Murine MoAb 1F5 (IgG2a) was produced in BALB/c mice and purified as previously described.<sup>13</sup> Antibody 1F5, along with the B1 antibody,<sup>13</sup> has been assigned to the CD20 (anti-Bp35) cluster group by the Second International Workshop on Human Leukocyte Differentiation Antigens.<sup>14</sup> The reactivity of antibody 1F5 with normal and malignant B cells has previously been reported.<sup>13,16,17</sup> MoAb 1F5 was purified from ascites by saturated ammonium sulfate precipitation followed by diethyl aminoethyl (DEAE)-Sephacryl (Pharmacia, Piscataway, NJ) column chromatography.<sup>16</sup> Testing of the purified antibody by Microbiological Associates (Bethesda, MD) has shown it to be free of bacterial, viral, or endotoxin contamination. Preclinical testing in two macaques (*M. fascicularis*) injected with 1F5 IV showed that this antibody was capable of eliminating circulating B cells and penetrating lymph nodes without causing any acute toxicity (J. Ledbetter, unpublished observations, 1983). A battery of normal human autopsy tissues was screened for reactivity with antibody 1F5 by an indirect immunoperoxidase method. No reactivity was seen with any tissue except those known to be rich in B lymphocytes (tonsils, lymph nodes, spleen). Tissues failing to bind 1F5 included heart, thyroid, adrenal, lung, muscle, kidney, testis, skin, colon, breast, and brain.

**Patient selection.** Adult patients with histologically confirmed B cell lymphomas shown by immunoperoxidase or immunofluorescence techniques to be reactive with the 1F5 antibody were eligible

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for this study if they had failed previous conventional therapy (chemotherapy and/or radiotherapy), if they had normal renal and hepatic function (creatinine  $<2.0$  mg/dL, bilirubin  $<1.5$  mg/dL), had evaluable disease, had not received any other treatment for  $\geq$  four weeks, had no other active medical problems, and signed an informed consent approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center.

**Study design.** Prestudy blood, marrow, and lymph node specimens were obtained and analyzed by conventional histopathology and by an indirect immunoperoxidase method (Vectastain, Vector Laboratories, Raritan, NJ) for evidence of tissue involvement with tumor cells capable of binding 1F5. Cell suspensions of these tissues were analyzed by two-color flow cytometry using a panel of fluorescein and phycoerythrin antibody conjugates<sup>13</sup> to determine the baseline immunologic phenotypes of the resident cell populations (see below). Intradermal skin testing with 10  $\mu$ g of antibody 1F5 in 0.1 mL of normal saline was performed, and no hypersensitivity responses were observed. Allopurinol (300 mg/d) was given throughout antibody administration. A bolus loading dose was given over one to two hours IV to rapidly achieve steady state serum antibody levels. The loading dose was calculated from the following equation: Loading dose =  $1.4 \times$  elimination half-time (in days)  $\times$  daily maintenance dose.<sup>18</sup> Preclinical studies of murine anti-CD20 antibody infusions in nonhuman primates (Ledbetter, unpublished data) and clinical trials of murine MoAbs administered to patients with graft-v-host disease (GVHD)<sup>19</sup> suggested an elimination half-time of 1.2 days, and this figure was used in calculating the loading doses. Patients were assigned a predetermined maintenance antibody dose that was diluted in 500 mL normal saline and administered by continuous IV infusion for five to ten days. (Patient 1 had premature discontinuation of his infusion after five days due to rapidly progressive lymphoma.) The maintenance antibody doses administered to the patients are summarized in Table 1. The dose escalation range was chosen to progress from safe low doses (5 mg/m<sup>2</sup>/d) known to be well tolerated for other MoAbs<sup>6,7,9,10,19</sup> to high doses (400 to 800 mg/m<sup>2</sup>/d), which we felt were more likely to result in good tissue penetration. We initially planned to escalate doses between patients. However, because of the absence of toxicity, poor penetration of low doses of antibody into patients 1 and 2, and slow patient accrual, doses were escalated progressively in patients 3 and 4 (from 10 mg/m<sup>2</sup>/d to 800 mg/m<sup>2</sup>/d) to achieve high circulating antibody levels that we felt would be more likely to achieve extravascular tissue penetration.

Table 1. Summary of Patient Characteristics

Patient	1	2	3	4
Age/Sex	42/M	64/M	63/M	45/M
Type of Lymphoma	DML	WDLL	DPDL	DHL
Stage	IVB	IVA	IVA	IVB
Total Dose of 1F5	52.4 mg	104.8 mg	1,032 mg	2,380 mg
Duration of Therapy	4.5 days	10 days	10 days	7 days
Response	Progression	Stable Disease	Minor Response	Partial Response

Abbreviations: DML, diffuse mixed small and large cell lymphoma; WDLL, diffuse well-differentiated lymphocytic lymphoma (small lymphocytic, working formulation); DPDL, diffuse, poorly differentiated lymphocytic lymphoma (diffuse small cleaved cell, working formulation); DHL, diffuse "histiocytic" lymphoma (diffuse large cell lymphoma, working formulation).

**Patient monitoring.** Pretreatment tests included a history and physical examination, relevant radiographic studies and computed tomographic scans, chemistry batteries, uric acid levels, complete blood cell counts (CBCs) and differentials, prothrombin time, partial thromboplastin time, serum complement levels (CH50, C4, C3), immune complex levels (C1Q binding assay), urinalysis, ECG, and cell surface marker analysis. Patients were examined twice daily during antibody infusion. Serial serum specimens for 1F5 levels and antimouse antibody levels, blood counts, chemistries, and blood specimens for surface marker studies were obtained four hours after initiation of 1F5 therapy and daily thereafter. Patients were discharged at the termination of antibody infusions. Blood samples were obtained on an outpatient basis for the above tests on days 1, 2, 7, and 21 after cessation of therapy and monthly thereafter. Serum complement and immunoglobulin levels were tested pretreatment and on days 1, 5, and 10 and then at roughly monthly intervals for six months.

**Response criteria.** Standard response criteria were employed as follows: Complete response-disappearance of all measurable and evaluable disease; Partial response; reduction by  $\geq 50\%$  of leukemic cell counts and  $\geq 50\%$  reduction in the size of a measurable lesion, and no increase in the size of any measurable or evaluable lesions or appearance of new lesions; Stable disease: Less than a partial response without an increase of  $>25\%$  in leukemic cell count and  $<25\%$  increase in any measurable lesion. Progression: Increase in leukemic cell count ( $>25\%$ ), appearance of new lesions, or an increase of 25% or greater in any measurable lesion.

**Measurement of free 1F5 and human antimouse antibody.** Serum 1F5 levels and human antimouse antibody levels (HAMA) were measured by solid phase competitive inhibition radioimmunoassay (RIA) as previously described.<sup>19</sup>

**Detection of cell-bound 1F5.** Assessment of tumor cell coating by infused antibody 1F5 was accomplished on serial specimens of peripheral blood, bone marrow, and lymph nodes by indirect immunoperoxidase and immunofluorescence techniques. Peripheral blood and bone marrow mononuclear cells were obtained by Ficoll-Hypaque (LSM, Litton Bionetics, Kensington, MD) density gradient centrifugation. Lymph node biopsies were divided in thirds: one portion was minced into a single cell suspension, another part was fixed in formalin for routine histologic staining, and another portion was frozen in liquid nitrogen for immunohistologic staining. Lymph node frozen sections were fixed to gelatin-coated glass slides and stained with rabbit antimouse immunoglobulin (Vectastain, Vector Laboratories, Raritan, NJ) using an indirect avidin-biotin technique. Pretreatment biopsies served as controls.

Cell suspensions of blood, bone marrow, and lymph nodes from antibody-treated patients were examined by flow cytometry (FACS IV, Becton Dickinson, Sunnyvale, CA) for the presence of surface 1F5 by using fluorescein-conjugated goat antimouse immunoglobulin (FITC-GAM1g; TAGO, Burlingame, CA). The mean fluorescence intensity of cells stained with FITC-GAM1g was compared to the intensity of cells incubated with excess 1F5 in vitro before staining to assess the saturation of binding sites in vivo. Relative CD20 surface antigen density was estimated for normal and malignant B cells by measuring the mean fluorescence intensity of cells stained in vitro with saturating quantities of fluorescein-conjugated anti-CD20 antibody after correcting for nonspecific fluorescence with a control reagent.<sup>13,17</sup>

Tumor cell surface antigen phenotypes were determined by both immunofluorescence and immunoperoxidase methods using peroxidase, fluorescein, or phycoerythrin conjugates of MoAbs 10.2 (anti-CD5), HB10a (anti-DR), G1-4 or 3E10 (antikappa), and 2C3 (anti- $\mu$ ) as previously described.<sup>13,17</sup> Serial monitoring of these tumor cell markers demonstrated that CD20-negative tumor cells were not

generated during 1F5 therapy by antigenic modulation (data not shown).

### CASE HISTORIES

Patient 1 was a 42-year-old man with stage IVB diffuse, mixed, small and large cell lymphoma who presented in 1982 with fever, diffuse lymphadenopathy, and hepatosplenomegaly. Previous therapy included CHOP (cyclophosphamide, adriamycin, vincristine, and prednisone) chemotherapy, intrathecal methotrexate, whole brain irradiation, splenectomy, sequential upper and lower hemibody irradiation, and four cycles of bleomycin, cytosine arabinoside, vincristine, procarbazine, and prednisone. He was referred for 1F5 therapy in July, 1984 because of refractory disease. He did not respond to low-dose 1F5 and was taken off study after five days of infusion because of progressive bone marrow (BM) and liver replacement with tumor. Salvage CHOP chemotherapy was given, but the patient died of progressive lymphoma on March 16, 1985.

Patient 2 is a 64-year-old man with stage IVA diffuse, small, lymphocytic lymphoma diagnosed by lymph node (LN) and BM biopsy in 1976. He received multiple chemotherapeutic regimens (CVP, CHOP, chlorambucil, and CCNU, etoposide, and methotrexate) with partial responses. He was referred for 1F5 serotherapy in December 1984, 11 months after his last course of chemotherapy. He did not respond to low dose 1F5 but had stable disease that did not require therapy until the summer of 1985 when he was begun on bleomycin, etoposide, BCNU, and Decadron, to which he remains partially responsive.

Patient 3 was a 63-year-old man with stage IVA diffuse, small, cleaved-cell lymphoma involving lymph nodes, marrow, and spleen. Previous therapy included splenectomy, chlorambucil, and CVP. He was referred for 1F5 therapy in December 1984, one month after his last cycle of CVP because of the development of refractory disease with rapidly progressive adenopathy and lymphocytosis ( $>30,000$  cells/ $\mu\text{L}$ ). He showed a minor response to intermediate dose 1F5 therapy. ProMACE/MOPP chemotherapy was given in January and February 1985 without response. A partial response occurred after therapy with high-dose cytosine arabinoside, but the patient died with marrow aplasia in July 1985.

Patient 4 was a 45-year-old man with sclerosing, diffuse large-cell lymphoma presenting in January 1983 with bowel and lymph node involvement. Therapy included eight cycles of CHOP, intrathecal methotrexate, involved field abdominal radiation, prophylactic cranial irradiation, and allogeneic marrow transplantation (in March 1984). He was referred for 1F5 therapy because of refractory lymphoma in late October 1985. He had been on dexamethasone (4 mg/d) for many months as symptomatic therapy for myalgias, and this was continued during serotherapy. After treatment with 1F5 there was a partial response that lasted six weeks. He then redeveloped progressive lymphoma and refused further treatment. He expired on December 21, 1985.

### RESULTS

**Serum 1F5 levels.** Circulating free antibody levels were detectable by RIA in all patients throughout the period of infusion (Fig 1). Patients 1 and 2 received MoAb doses of 5 mg/ $\text{m}^2$ /d and consistently had 1F5 serum concentrations of 0.3 to 1.0  $\mu\text{g}/\text{mL}$ . Patients 3 and 4 received escalating antibody doses and had corresponding increases in 1F5 levels. For comparable antibody doses the patients with circulating antigen-positive tumor cells (1 and 3) had lower serum levels of 1F5 than the patients who did not have significant numbers of circulating malignant lymphocytes (2 and 4, see Fig 1), probably reflecting the effect of antibody

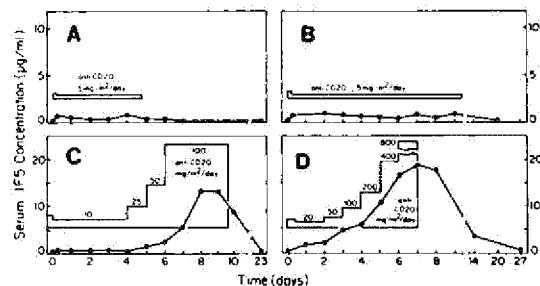


Fig 1. Serum 1F5 concentrations in patients receiving continuous infusions of antibody. MoAb 1F5 concentrations were determined by solid phase radioimmunoassay. The doses of 1F5 infused in each patient are indicated by the superimposed stippled bars. (A), patient 1; (B), patient 2; (C), patient 3; (D), patient 4.

binding to tumor cells. Antibody remained detectable in the serum for as long as three weeks after termination of infusion in patient 4 (peak concentration 190  $\mu\text{g}/\text{mL}$ ). However, 1F5 levels fell into the undetectable range within three days of termination of infusion in patients 1 and 2 (peak levels 1  $\mu\text{g}/\text{mL}$ ) and within two weeks in patient 3 (peak level 13.4  $\mu\text{g}/\text{mL}$ ). Rough estimates of the serum elimination half-times were calculated to be 24 hours for patient 1 (from 0.93  $\mu\text{g}/\text{mL}$  to .22  $\mu\text{g}/\text{mL}$  in the 48 hours after termination of infusion), 42 hours for patient 3 (from 13.4  $\mu\text{g}/\text{mL}$  to 9.0  $\mu\text{g}/\text{mL}$  in 24 hours), and 52 hours in patient 4 (from 179 to 35.6  $\mu\text{g}/\text{mL}$  over five days). Data for patient 2 were insufficient for estimation of a serum half-life. These elimination half-times are in good agreement with previous studies of murine anti-CD20 antibodies in nonhuman primates (J. Ledbetter, unpublished results) and studies of murine anti-T cell antibodies in patients with GVHD.<sup>19</sup>

**CD20 antigen density on tumor cells.** Table 2 summarizes the relative Bp35 surface antigen densities on patient lymphoid cells from blood, bone marrow, and lymph nodes as determined by direct immunofluorescent analysis.<sup>13,17</sup> The density of this antigen on normal B lymphocyte populations is also listed for comparison. Patients 1 and 4 had CD20 densities on their malignant cells comparable to those seen on normal, resting B lymphocytes (eg, peripheral blood B cells and tonsil mantle zone B cells<sup>13</sup>). Patient 3 had a much higher surface antigen density on his lymphoma cells, comparable to that observed on normal, activated B cells (tonsil germinal center cells<sup>13</sup>). Patient 2 had a very low Bp35 antigen density on his lymph node and bone marrow tumor cells with a mean fluorescence intensity only 3.5 times higher than control cell populations lacking the antigen. (This degree of staining was unequivocally greater than control, however.) Patients 2 and 4 had negligible numbers of circulating tumor cells morphologically, confirming the negligible staining with FITC-1F5 seen by immunofluorescence (nearly all circulating lymphocytes were T cells in these patients). Of interest, the bone marrow of patient 4 was grossly involved with tumor but failed to bind FITC-1F5, suggesting that an antigen-negative tumor cell variant was responsible for infiltration of this tissue. With this single exception, the different sites of lymphomatous involvement within a given patient showed similar CD20 antigen densi-



**Table 2. Relative CD20 Antigen Densities on Normal and Malignant Lymphoid Cells**

Cell Type	Relative Antigen Density*
<b>Normal Tissues</b>	
1. Peripheral Blood T cells	2†
2. Peripheral Blood B cells	82
3. Tonsil Mantle Zone B cells	70
4. Tonsil Germinal Center B cells	256
<b>Lymphoma Patients</b>	
1. Patient 1‡	
a. Blood lymphocytes	96
2. Patient 2	
a. Bone marrow	7
b. Lymph node cells	7
3. Patient 3	
a. Blood lymphocytes	301
b. Bone marrow cells	301
c. Lymph node cells	235
4. Patient 4	
a. Blood lymphocytes (uninvolved)	2
b. Bone marrow cells	2
c. Lymph node cells	84

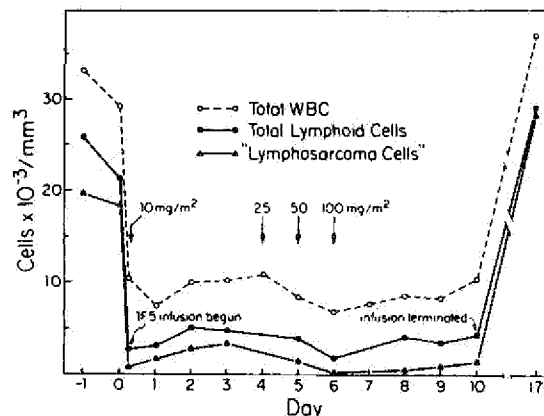
\*Expressed as the linear channel number of the mean fluorescence intensity measured on a FACS IV cell sorter for cells stained with saturating concentrations of fluorescein-conjugated anti-CD20 antibody by the method of Ledbetter and Clark.<sup>12</sup>

†Negative control (unstained) cells also showed a mean fluorescence intensity of 2.

‡Patient 1 had an unascrable marrow and no accessible adenopathy, so immunofluorescent studies were done solely on circulating malignant cells.

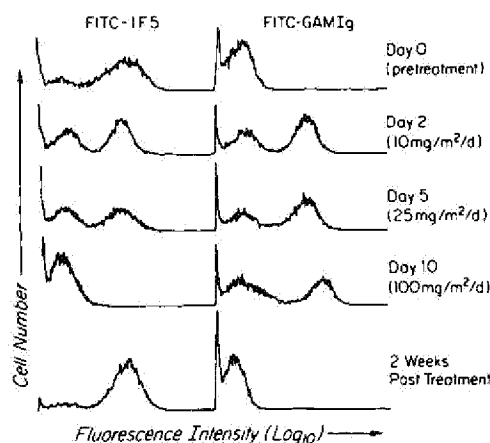
ties. No definitive conclusion regarding the clinical responsiveness of tumors bearing different surface CD20 densities is possible because of the small number of patients treated and variable antibody doses administered.

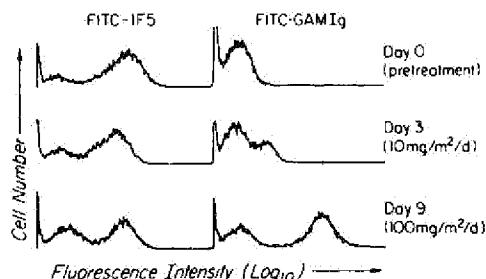
**Effects of 1F5 on peripheral blood lymphocytes.** Two patients (1 and 3) had appreciable numbers of circulating malignant cells. In both patients antibody administration resulted in an immediate decrease in the number of circulating tumor cells (assessed by morphological criteria and by surface immunologic phenotypes). Patient 1 had an 86% decline in the number of blood lymphoma cells (from  $1.27 \times 10^3/\mu\text{L}$  to  $0.18 \times 10^3/\mu\text{L}$ ) within four hours of institution of 1F5 therapy. Patient 3 had a 91% decrement in circulating tumor cells (from  $18.21 \times 10^3/\mu\text{L}$  to  $1.61 \times 10^3/\mu\text{L}$ ) in the same brief time interval (Fig 2). These effects were obtained with low doses of antibody in both patients ( $5 \text{ mg}/\text{m}^2$  and  $10 \text{ mg}/\text{m}^2$ , respectively) and were sustained throughout the entire period of infusion (five and ten days). Flow cytometry of circulating PBL stained with FITC-GAM1g demonstrated saturation of 1F5 antibody binding sites on tumor cells in both patients (although complete saturation in patient 3 was only achieved at the higher dose of  $100 \text{ mg}/\text{m}^2/\text{d}$ , Fig 3). Serial tumor cell surface-antigen phenotyping (using two-color immunofluorescence with reagents recognizing other tumor-associated antigens [see Materials and Methods]) demonstrated that antigenic modulation did not occur (data not shown). In both patients termination of 1F5 therapy was accompanied by a rapid reappearance of

**Fig 2. Depletion of circulating lymphoma cells in patient 3 during infusion of MoAb 1F5.**

circulating tumor cells that reached pretreatment levels within two to three days (Fig 2).

**Effects of 1F5 on bone marrow tumor cells.** Patients 1, 2, and 3 had evaluable marrow involvement with lymphoma. In patients 1 and 2, antibody doses of  $5 \text{ mg}/\text{m}^2/\text{d}$  were not sufficient for saturation of 1F5 antibody binding sites on tumor cells in the marrow. Patient 3 received escalating doses of 1F5 in conjunction with serial marrow aspirations to estimate the amount of antibody required for coating of tumor cells in the marrow. Serial fluorescence histograms (Fig 4) clearly showed that an antibody dose of  $10 \text{ mg}/\text{m}^2/\text{d}$  was insufficient (4% saturation of Bp35 binding sites) but that  $100 \text{ mg}/\text{m}^2/\text{d}$  could produce significant coating (61% saturation of Bp35 binding sites) of marrow tumor cells.

**Fig 3. In vivo labeling of circulating tumor cells with 1F5 antibody in patient 3 as assessed by serial flow cytometry of peripheral blood lymphocytes with FITC-GAM1g (to detect mouse antibody 1F5 bound to tumor cells in vivo) and FITC-1F5 (to detect unoccupied Bp35 [CD20] binding sites). Pretreatment PBL stained brightly with FITC-1F5 because of abundant free CD20 sites on circulating lymphoma cells. Serial histograms on days 2, 5, and 10 revealed coating of PBL with 1F5 (detected with FITC-GAM1g). Saturation of binding sites is shown on day 10 by absence of unoccupied Bp35 receptors capable of binding FITC-1F5. By two weeks posttreatment, bound murine MoAb was no longer detectable on PBL.**



**Fig 4.** Penetration of MoAb 1F5 into bone marrow of patient 3 during serotherapy as assessed by flow cytometry of mononuclear cells from BM aspirates using FITC-GAMlg (to detect cell-bound 1F5) and FITC-1F5 (to detect unbound Bp35 sites).

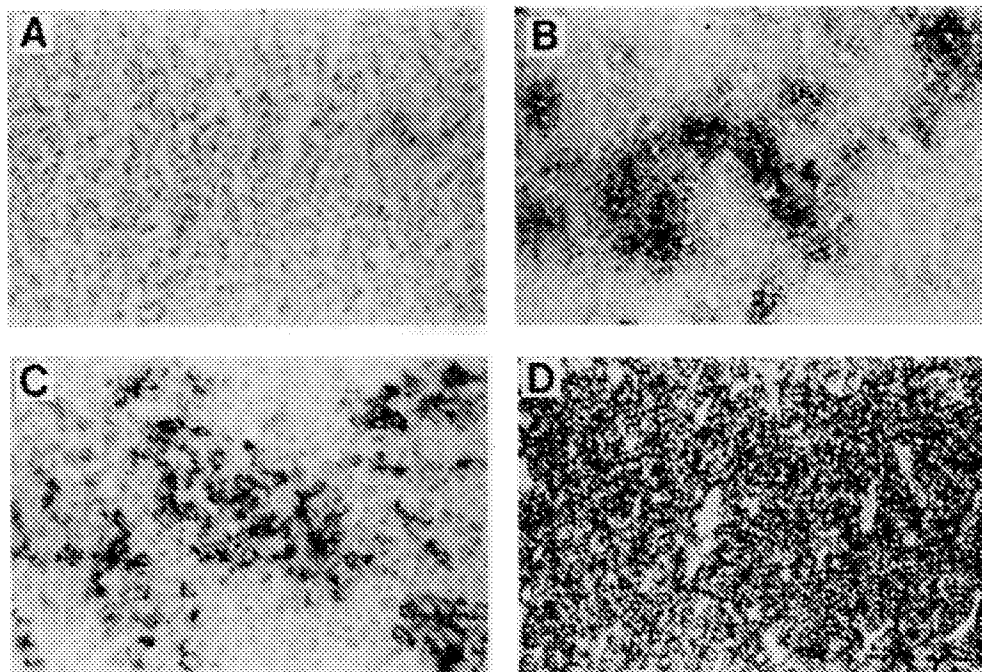
Regression of marrow lymphoma was not seen in any of these three patients.

The marrow of patient 4 was unusual in that it appeared to contain tumor cells that did not express the Bp 35 antigen. Although marrow aspirates and biopsies contained unequivocal large cell lymphoma, no tumor cells reactive with FITC-1F5 were detected by flow cytometry. In contrast, tumor cells in cervical and inguinal lymph nodes had the same morphology as the cells in the marrow but reacted strongly with antibody 1F5 as assessed by both immunoperoxidase and immunofluorescence techniques (see Table 2). As would be anticipated, infusion of antibody 1F5 had no effect on the antigen-negative tumor cells in the marrow of this patient.

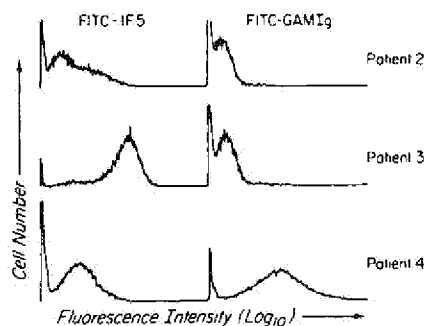
**Effects of 1F5 on lymph nodes.** Patients 2, 3, and 4 had evaluable adenopathy that was biopsied before treatment and on the last day of antibody infusion. Immunoperoxidase and immunofluorescent analyses showed no penetration of antibody 1F5 into the nodes of patient 2 (who received 105 mg over ten days). There was minor perivascular penetration detectable only by immunoperoxidase methods in patient 3 (1,032 mg over ten days). In contrast, significant coating of tumor cells detectable by both immunoperoxidase and immunofluorescence (Figs 5 and 6) was present in patient 4 (2,380 mg over seven days) with 69% saturation of available binding sites. In vitro studies showed that an ambient 1F5 antibody concentration of 24  $\mu$ g/mL was necessary to achieve 69% saturation of cell-surface binding sites. Since the serum 1F5 concentration in patient 4 at the time of his lymph node biopsy was approximately 190  $\mu$ g/mL, we estimate that a 1F5 antibody gradient of 8:1 existed between serum and lymph node interstitial fluid.

No clinical response was observed in the nodes of patient 2. Some inguinal nodes regressed by 25% in patient 3, but most lymph nodes were unaffected. There was marked regression of all nodes in patient 4 with a calculated >90% reduction in tumor burden (Fig 7). Of note, the diminution of LN size did not begin until day 5 of antibody infusion, and progressive node shrinkage continued for three weeks after cessation of 1F5 infusion. The response duration was brief, however, with regrowth of LN occurring six weeks after therapy.

**Overall clinical response.** Patient 1 had diminution of



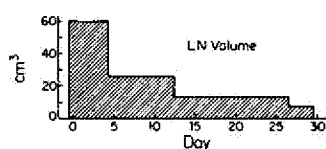
**Fig 5.** Indirect immunoperoxidase staining of lymph node frozen sections with GAMlg to detect in vivo labeling of tumor cells during murine MoAb 1F5 serotherapy (original magnification  $\times 250$ ). (A) Patient 2: LN biopsy performed on day 10 of therapy while receiving 5 mg/m<sup>2</sup>/d of antibody 1F5. Negligible staining indicates absence of penetration of LN by antibody at this dose. (B) Patient 3: LN biopsy performed on day 9 of therapy while receiving 100 mg/m<sup>2</sup>/d of 1F5. Staining of tumor cells in perivascular locations is present. (C) Patient 4: LN biopsy performed on day 10 of therapy while receiving 800 mg/m<sup>2</sup>/d of 1F5. Peroxidase staining is appreciable at this dose, although the distribution remains heterogeneous. (D) Saturation of 1F5 binding sites in patient 3 by in vitro incubation of an LN section with excess 1F5 antibody. The section shown in this figure was from the same LN biopsy depicted in Fig 4B.



**Fig 6.** Analysis of 1F5 penetration into LN during serotherapy assessed by flow cytometry of LN suspensions. FITC-GAM1g was used to detect in vivo coating of tumor cells with antibody 1F5. Patient 2 had only a small subpopulation of LN cells possessing the Bp35 antigen (shown with FITC-1F5). No penetration of 1F5 into LN could be shown with FITC-GAM1g. Virtually all LN cells in patient 3 possessed the Bp35 antigen and stained intensely with FITC-1F5, but poor in vivo penetration of 1F5 had occurred as shown by absence of FITC-GAM1g staining. (Immunoperoxidase staining of LN sections was capable of demonstrating some penetration of 1F5 into this LN, however [see Fig 5B]). Patient 4 had significant penetration of 1F5 into LN as shown by staining with FITC-GAM1g.

circulating tumor cells, but progressive liver and marrow lymphoma required premature termination of 1F5 therapy and institution of salvage chemotherapy. Patient 2 had no response of his evaluable marrow or LN disease. Patient 3 had a minor response consisting of transient 90% reduction of circulating tumor cells and 25% shrinkage of some but not all LN but no response in the marrow. Patient 4 had a partial response consisting of 90% reduction of all evaluable LN.

**Toxicity.** No clinically significant toxicity was observed in any of our patients. Patients 1 and 4 had asymptomatic, intermittent, low-grade fever (38 to 39 °C) lasting six days and two hours, respectively. Transient decrements of platelet and neutrophil counts to 50% to 75% of baseline levels were observed in all four patients. These changes were rapidly reversible in all cases except patient 1, where progressive cytopenias were clearly due to marrow replacement with tumor. In the other three instances, the blood counts stabilized after one to two days and often demonstrated some recovery, even before discontinuation of antibody infusion. No bleeding or infectious episodes occurred during antibody treatment. No allergic, pulmonary, renal, hepatic, or cutaneous sequelae occurred. Renal function as measured by 24-hour creatinine clearance was unchanged after completion of antibody therapy. Complement consumption was observed in patients 1 (40% reduction of CH<sub>50</sub> and C4 levels) and 3 (97% reduction of CH<sub>50</sub>, 94% reduction of C4 levels), but circulating immune complexes could not be detected at any time in any patient.



**Fig 7.** Reduction in tumor burden in patient 4. A 90% reduction in LN volume was observed over a four-week period.

**Effects of 1F5 on normal B cells.** Attempts to monitor numerical changes and functional alterations of normal B cells in patients receiving 1F5 serotherapy (by assessing proliferative responses to polyclonal B cell mitogens and in vitro Epstein-Barr virus (EBV)-induced immunoglobulin synthesis) were precluded by the extremely low numbers of normal B cells that could be harvested from these patients following antibody infusion. The paucity of B cells was due to two factors: baseline deficiency of normal B lymphocytes (a common feature of advanced, refractory B cell malignancies) and depletion of normal as well as malignant B cells by 1F5 therapy (which has also been documented in normal nonhuman primates infused with anti-CD20 antibodies, [Jeffrey Ledbetter, unpublished results]). Quantitative immunoglobulin levels were carefully monitored in all patients before, during, and for up to five months after 1F5 therapy. Baseline hypogammaglobulinemia was present in patients 1 and 3, but no patient demonstrated a decline in any subclass of immunoglobulin consequent to 1F5 therapy.

**Human antimouse antibody levels.** IgM HAMA were undetectable by RIA in all patients. Low levels of IgG HAMA (2 × pretreatment control levels) became detectable five months after serotherapy in patient 1 only.

## DISCUSSION

This report summarizes our findings in four patients with refractory malignant B cell lymphomas treated with MoAb 1F5 (anti-CD20) by continuous IV infusion for five to ten days. Our study differs from previous serotherapy trials of hematologic malignancies by employing an antibody directed against a nonmodulating antigen. This feature allowed us to maintain continuous high serum antibody levels without inducing the tumor refractoriness generally encountered with modulating antigens.<sup>8-11,20</sup> The continuous infusion mode of administration allowed delivery of very high doses of antibody (up to 800 mg/m<sup>2</sup>/d in patient 4) without the significant pulmonary toxicity that is often observed following bolus injection of high MoAb doses.<sup>8,10,21</sup> Serial kinetic measurements revealed a dose-dependent relationship between the amount of antibody infused and the concentration of free MoAb in the blood stream. We found that even low doses of 1F5 (5 to 10 mg/m<sup>2</sup>/d) were capable of depleting circulating tumor cells from the blood stream analogous to observations made using murine MoAbs T101 (for chronic lymphocytic leukemia) and J5 (for acute lymphocytic leukemia).<sup>9-11,20</sup> However, in contrast to the studies with modulating antibodies T101 and J5 given by prolonged or repeated administration, the responses induced by the nonmodulating 1F5 were sustained throughout the duration of the infusion (five to ten days).

Although small doses of 1F5 sufficed to deplete circulating tumor cells, penetration of antibody into extravascular sites such as bone marrow and lymph nodes proved to be much more problematic. Intravenous administration of 400 to 800 mg/m<sup>2</sup>/d was required to achieve 69% saturation of binding sites on lymph node tumor cells. Even at these doses the intranodal distribution of antibody was heterogeneous. The immunoperoxidase staining patterns observed in LNs

suggested passive diffusion of antibody down a concentration gradient from small blood vessels into the LN parenchyma. The clinical responsiveness observed appeared to correlate with IF5 dose administered, peak-serum MoAb concentration achieved, and degree of extravascular tissue penetration obtained. A total dose of 52.4 mg was associated with progressive disease (patient 1), 104.8 mg resulted in stable disease in patient 2, 1,032 mg caused a minor response in patient 3, and 2,380 mg produced a partial response in patient 4.

These observations have been made on a very small group of patients, each of whom had a different type of B cell lymphoma. The degree to which our findings might be applicable to other lymphoma patients is unclear, since each patient has a different tumor burden and distribution, different numbers of circulating malignant cells acting to absorb infused MoAb, and different surface densities of the Bp35 antigen. Consequently, the dose levels and kinetic data found in our patients can only serve as a rough guideline for the management of other patients with this antibody. Generalizations from our findings with IF5 to other MoAbs should be made with caution in light of recent studies demonstrating dramatic, unpredictable kinetic and functional differences among different antibodies recognizing the same antigen.<sup>22</sup>

The toxicity seen in these four patients was insignificant. Minor fever and moderate cytopenias were the only adverse effects observed despite administration of massive doses of antibody IF5 to patients 3 and 4. Since neither platelets nor neutrophils express the Bp35 antigen or label with FITC-IF5, the exact mechanism for the decrement in neutrophil and platelet counts is uncertain. It is of interest that similar decrements in blood counts have also been observed in patients treated with anti-idiotypic antibodies.<sup>8</sup> Small quantities of antibody might be absorbed via Fc receptors to these cells, which may then be removed from the circulation by the reticuloendothelial system. Although the development of HAMA has been a major problem in some reported series,<sup>8,23</sup> antihuman antibodies were detected in only one of our four patients, and in this patient they did not appear until five months after IF5 therapy was completed. These findings are in accord with other studies demonstrating that patients with B cell malignancies undergoing monoclonal serotherapy seldom develop HAMA, whereas patients with T cell malignancies or solid tumors receiving similar treatment often develop antihuman antibodies.<sup>24,25</sup>

Previous trials of MoAb serotherapy have also generally encountered minimal toxicity.<sup>3</sup> The major adverse events described to date involved anaphylactoid reactions in patients with large circulating tumor cell burdens and/or high circulating antigen levels given high doses of antibody by rapid bolus injection.<sup>8,10,21</sup> These episodes have been ascribed to pulmonary leukostasis resulting from sequestration of antibody-coated tumor cells in the pulmonary vasculature leading to wheezing, dyspnea, and hypotension. The absence of circulating antigen and the prolonged duration of antibody administration in our trial were mitigating factors that probably helped avoid these untoward sequelae in our patients.

The relative merits of continuous infusion of MoAbs

compared with intermittent bolus therapy remain debatable. For many antibodies continuous infusion is not feasible because of antigenic modulation. In such circumstances intermittent therapy is necessary to allow regeneration of cell surface antigen. The bolus method is less cumbersome than continuous infusion and achieves higher peak MoAb concentrations for equivalent doses. Whether the maintenance of uniform high-circulating antibody levels and reduction in toxicity achievable with continuous infusion are sufficiently advantageous to offset the inconveniences remains unanswered. Nevertheless, maintenance of steady state antibody levels in this trial has afforded an advantageous setting for kinetic measurements and for the assessment of the serum concentrations required for MoAb penetration into the extravascular space.

The mechanisms by which unmodified MoAbs might cause elimination of tumor cells *in vivo* remain controversial. Most workers currently view antibody-dependent cellular cytotoxicity and reticuloendothelial system phagocytosis of MoAb-coated cells as the most likely processes involved.<sup>3,26</sup> Murine MoAbs (including IF5) fix human complement poorly *in vitro*, and consequently complement-mediated tumor cell lysis is not thought to be of major significance *in vivo*. The significant consumption of complement in two of our four patients was unexpected and suggests a possible role for complement in eliminating tumor cells.

The short, incomplete responses obtained with serotherapy using unmodified MoAbs have been of minimal clinical benefit (with the notable exception of the patient described by Miller et al).<sup>7</sup> Consequently, innovative MoAb administration schedules, testing of new antibodies, and administration of antibody conjugates will be necessary if the promise of monoclonal serotherapy is to be realized. Badger et al have already convincingly demonstrated cures of lymphomas in mice treated with radioiodinated MoAbs in a setting in which unmodified MoAbs were ineffective.<sup>27</sup> Recent clinical reports of responses in patients with Hodgkin's disease or hepatoma treated with radioiodinated antiferritin antibodies suggest that this approach will also be useful in man.<sup>28,29</sup> Radiolabeled MoAbs can potentially kill not only the tumor cells to which they bind but could also kill neighboring cells that do not bind antibody by virtue of poor tissue penetration, antigenic modulation, or somatic mutation ("antigen-negative variants"). Toxin-antibody conjugates are similarly promising,<sup>30</sup> although antigens such as CD20, which are not endocytosed after ligand binding,<sup>31</sup> might not be good targets for this approach, since immunotoxin internalization is generally required for cell killing.<sup>32</sup> The findings of our current pilot study should assist in the rational design of subsequent trials employing such radiolabeled or toxin-conjugated immunotoxins.

#### ACKNOWLEDGMENT

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# Value of Monoclonal Anti-CD22 (p135) Antibodies for the Detection of Normal and Neoplastic B Lymphoid Cells

By D.Y. Mason, H. Stein, J. Gerdes, K.A.F. Pulford, E. Ralfkiaer, B. Falini, W.N. Erber, K. Micklem, and K.C. Gatter

Two monoclonal antibodies (To15 and 4KB128) specific for the B cell-associated CD22 antigen (135,000 mol wt) are described. On immunoenzymatic analysis of cryostat tissue sections, these antibodies strongly label both mantle zone and germinal center B lymphoid cells in secondary lymphoid follicles (and also scattered extrafollicular lymphoid cells) but are unreactive with other cell types (with the exception of weak reactivity with some epithelioid histiocytes). These reactions differ from those of monoclonal antibodies B1 and B2 (anti-CD20 and CD21) but are similar to those of the pan-B antibody B4 (anti-CD19). One of the anti-CD22 antibodies (To15) has been tested extensively by immunoenzymatic labeling on >350 neoplastic lymphoid and hematological samples. The CD22 antigen was found in tissue sections in most B cell-derived neoplasms, the major

exceptions being myeloma (all cases negative) and a small proportion of high-grade lymphoma (6% of cases negative). In cell smears, the antigen could be found on neoplastic cells in most B cell lymphoproliferative disorders, including common acute lymphoblastic leukemia (ALL) (90% positive) and B cell chronic lymphocytic leukemia (CLL) (89% positive). We conclude that anti-CD22 antibodies are of value for identification of human B cell lymphoproliferative disorders (especially when used in conjunction with anti-CD19 antibodies). Previous reports that the CD22 antigen is absent from many B cell neoplasms are probably due to its being expressed within the cytoplasm of immature B cells rather than on their surface.

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**W**IDESPREAD use has been made in recent years of monoclonal antibodies for the immunocytochemical identification of lymphoid cell populations in human tissue and blood samples. The first of these reagents to be characterized were directed against T cell antigens, and at the 1st International Workshop on Human Leucocyte Differentiation Antigens (held in Paris in 1982) eight different T cell-associated molecules were defined.<sup>1</sup> Following the convention established at that Workshop, these molecules are prefixed by the letters CD and listed from 1 to 8.

An equivalent number of B cell-associated markers was not defined at the first Workshop, the only categories emerging at that time being CD9 (a 24,000 mol wt molecule shared between B cells and renal tissue) and CD10, the Common-ALL antigen (CALLA). A number of new B cell-associated molecules were, however, defined in the second Workshop (held in Boston in 1984) and numbered CD19 through 24.<sup>2</sup> Several of these appear to be restricted to B cells, whereas others are shared between B cells and dendritic reticulum cells (the antigen-presenting cell found in B cell follicles).<sup>3</sup>

Although these B cell-associated molecules are clearly defined entities, little information is available on their relative value as markers in the immunocytochemical detection of human B cell neoplasms. In the present article, we describe our experience in using two monoclonal antibodies against the 135,000 mol wt CD22 antigen for the detection of neoplastic B cells in cryostat sections and cell smears. It is

evident from this study that anti-CD22 antibodies can be confidently used as pan-B reagents for identification of B cell neoplasms, since they react with most such tumors and are unreactive with T cell-derived neoplasms.

## MATERIALS AND METHODS

### Tissues

Human tissue biopsies were obtained fresh from the Histopathology Department of the University Hospital, Kiel, and from the John Radcliffe Hospital, Oxford, England. Routine tonsillectomy samples were obtained from the Ear, Nose, and Throat Departments of these hospitals. Histological diagnosis was based on examination of paraffin sections stained with hematoxylin and eosin and/or Giemsa, lymphoid neoplasms being classified according to the Kiel system. Immunohistological phenotyping following immunoenzymatic staining of cryostat sections (described later) was performed as described elsewhere.<sup>4-6</sup>

### Cell Samples

Smears of peripheral blood and bone marrow samples from the Hematology Department of the John Radcliffe Hospital and of normal blood from healthy laboratory personnel were stored at -20°C until they were labeled immunoenzymatically. In addition, cytocentrifuge preparations of Triosil-Ficoll-separated mononuclear cells were stored in the same way. Tonsil cell suspensions were prepared and, in some experiments, enriched in follicular dendritic reticulum cells (FDRCs), as described previously.<sup>7</sup>

In some experiments, cytocentrifuge preparations were made from normal peripheral blood mononuclear cells that had been rosetted with neuraminidase-treated sheep RBCs.

### Immunocytochemical Reagents

Details of the monoclonal antibodies used in this study and of the immunoenzymatic reagents (eg. PAP, APAAP, peroxidase conjugates) are given in previous publications.<sup>5,8,9</sup> Antibodies B1, B2 and B3 were obtained from Dr L. Nadler and from Coulter.

### Immunoenzymatic Labeling Procedures

Tissue sections and cell smears were labeled by a two-stage or three-stage indirect immunoperoxidase procedure,<sup>10</sup> by the PAP immunoperoxidase technique,<sup>9</sup> or by the APAAP immunoalkaline phosphatase technique.<sup>11,12</sup>

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### Production of Monoclonal Antibodies to the CD22 B Cell-Associated Antigen

Two antibodies of this specificity were prepared and used in this study.

**Antibody To15.** A cell fusion experiment was performed using spleen cells from a Balb-c mouse immunized with a potassium iodide extract of normal human tonsil cell membranes.<sup>13,14</sup> Culture supernatants were screened by immunoperoxidase labeling of tonsil cryostat sections,<sup>15</sup> and one clone (To15) reacting selectively with B cell follicles was cloned and grown by conventional procedures.<sup>14</sup>

**Antibody 4KB128.** Antibody 4KB128 was obtained (by the same hybridoma technique) using spleen cells from a mouse previously immunized with neoplastic cells from a case of hairy cell leukemia.<sup>16</sup>

### Immunoprecipitation

Immunoprecipitation experiments were performed by conventional procedures, using surface-iodinated human tonsil cells and rabbit anti-mouse Ig bound to Staphylococcal protein A as precipitating reagent. Electrophoresis in sodium dodecyl sulfate (SDS) polyacrylamide gel and autoradiography were performed by conventional techniques.

### Purification of Antigen

Membranes extracted from frozen hairy cell leukemia spleen in 2.5% Tween 40 were solubilized in 2% Nonidet P40, 20 mmol/L of Tris-HCl pH 7.5 containing protease inhibitors.<sup>17</sup> The detergent extract was precleared by passage over bovine serum albumin (BSA) coupled to Sepharose 4B, and 25 mL (equivalent to 20 g of original tissue) was then passed down a 5 mL Sepharose 4B:4KB128 antibody column (antibody 4 mg/mL of Sepharose) at 1 mL/h. After being washed with 50 mL of 250 mmol/L of NaCl in 1% Nonidet P40, 20 mmol/L of Tris-HCl pH 7.5 at 2 mL/h, bound antigen was eluted with a 40-mL pH 7.5 linear gradient at 2 mL/h. Fractions were precipitated with 10% trichloroacetic acid (TCA), washed with acetone, solubilized in SDS-urea, and analyzed by SDS polyacrylamide gel electrophoresis (PAGE) using a 7.5% gel and Coomassie blue staining.

## RESULTS

### Production of Monoclonal Antibodies To15 and 4KB128

Supernatant from two hybridoma cell cultures, To15 and 4KB128, reacted selectively with B cell follicles in lymphoid tissue, and the same pattern was maintained during cloning and subsequent growth of these cell lines.

### Mol Wt of Antigen Detected by Antibodies To15 and 4KB128

Antibodies To15 and 4KB128 both precipitated a polypeptide chain with a mol wt under reducing conditions of 125,000 to 135,000. In some experiments, this material appeared as a doublet of two bands of only slightly different mol wts. It was also possible to isolate the CD22 antigen from human tissue using Sepharose antibody immunoabsorbents (Fig 1). This material migrated with the same mol wt as that of the radiolabeled band detected by immunoprecipitation.

### Immunocytochemical Labeling of Peripheral Blood Cells

**Immunoenzymatic staining of cell smears.** Antibody To15 labeled 15% of lymphoid cells (range 10% to 25%) in

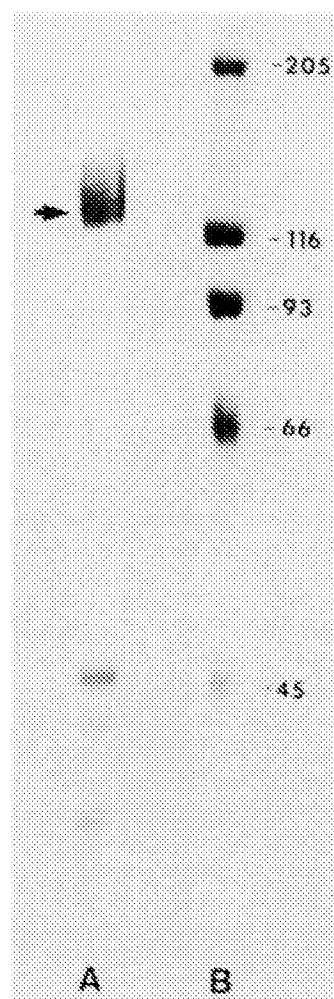


Fig 1. Demonstration of the specificity of antibody 4KB128 for the CD22 antigen by elution from an immunoabsorbent column (described in text). In track A, the prominent protein band (arrow) of ~125,000 mol wt represents the CD22 antigen. Small amounts of contaminating protein of lower mol wt are near the bottom of the gel. Track B shows the mol wt (in kD) of marker proteins run on the same gel. The mol wt of the CD22 antigen in this experiment was lower than the conventionally quoted figure (135,000) but in keeping with results reported by other authors<sup>24</sup> (Coomassie blue staining).

blood smears from 15 normal subjects. Antibody 4KB128 (tested on a smaller number of samples) labeled essentially the same number of lymphoid cells. Granulocytes, monocytes, RBCs, and platelets were unstained. In cytocentrifuge preparations of peripheral mononuclear cells rosetted with sheep erythrocytes, antibodies To15 and 4KB128 reacted selectively with nonrosetting lymphocytes.

### Immunohistological Labeling of Human Tissue Sections

**Normal tissue.** The two anti-CD22 antibodies labeled B cell follicles with moderate to strong intensity (depending on the labeling procedure used) staining both mantle zone lymphocytes and germinal center cells. In centrifuged preparations of tonsil-cell suspensions enriched for FDRs, antibody To15 labeled these cells weakly, although this could not

be discerned (because of lymphoid cell reactivity) in tissue sections.

The two anti-CD22 antibodies were compared with the pan-B reagents B1, B2, and B4 (anti-CD20, 21, and 19, respectively). Neither B1 nor B2 labeled B cell follicles as extensively as, or gave reactions of comparable intensity to, the two anti-CD22 reagents. Both B1 and B2 stained follicle mantle zone lymphocytes more weakly than germinal centers, and much of the germinal center staining was in a meshwork pattern in germinal centers characteristic of FDRCs (particularly marked with antibody B2). In contrast, antibody B4 was closely similar in its staining reactions to the anti-CD22 reagents, labeling both mantle zones and germinal centers.

In addition to B cell follicles, antibodies To15 and 4KB128 stained scattered small cells in interfollicular and paracortical regions. The two antibodies were unreactive with all nonlymphoid tissue and organs tested (skin, lung, stomach, small and large intestine, kidney, liver, salivary and thyroid gland, urinary bladder, testis, and central and peripheral nervous tissue), with the exception of weak staining of epithelioid histiocytes in granulomatous lesions. Antibody B1 also reacted with epithelioid histiocytes, usually more intensely than the anti-CD22 reagents.

**Neoplastic tissues.** One of the two anti-CD22 antibodies (To15) was tested against a wide range of neoplasms (Table 1). It labeled most human B cell neoplasms, the principal exceptions being myeloma (consistently negative) and a few high-grade B cell lymphomas (4 negatives in 64 cases). The strength of labeling was usually of moderate intensity and could be enhanced markedly by use of the three-stage immunoperoxidase technique<sup>10</sup> or the "enhanced" APAAP procedure.<sup>9</sup> The strongest reactions were seen in hairy cell leukemia and B cell prolymphocytic leukemia. The intensity of labeling of cases of common acute lymphoblastic leukemia (common ALL) and B cell chronic lymphocytic leukemia (CLL) varied widely; a number of positive cases showed only weak labeling. Nevertheless, most cases in each of these two disorders gave unequivocally positive reactions.

Other neoplasms analyzed with antibody To15 (Table 1), were unreactive (including 56 cases of T cell lymphoproliferative disease) with the exception of a minority of cases of acute myeloid leukemia. These reactions were usually weak; in one case of acute monocytic leukemia, however, the neoplastic cells were relatively strongly labeled. The other anti-CD22 antibody (4KB128) was tested on a smaller number of neoplasms and gave reactions closely similar to those of antibody To15.

#### DISCUSSION

Both of the antibodies used in this study appear to recognize the B cell-associated molecule designated CD22. This is evidenced both by the results of immunoprecipitation and affinity chromatography experiments from this laboratory and by similar experiments and statistical analysis of FACS data in the 3rd International Workshop on Human Leucocyte Differentiation Antigens.<sup>18</sup> The observation that in some experiments two polypeptide chains of similar mol wt

**Table 1. Immunocytochemical Staining of Human Neoplasms With Antibody To15 (Anti-CD22)**

Neoplasm	No. Tested	No. Positive
<b>B cell derived</b>		
Chronic lymphocytic leukemia	53	49
Prolymphocytic leukemia	7	7
Hairy cell leukemia	28	28
Lymphoplasmacytoid lymphoma/Waldenström's	9	8
Multiple myeloma	26	0
Centroblastic-centrocytic lymphoma*	22	22
Centrocytic lymphoma	18	18
Centroblastic lymphoma†	23	21
Lymphoblastic lymphoma, Burkitt-type	9	9
Lymphoblastic lymphoma, non-T, CALLA-positive	14	14
Immunoblastic lymphoma†	18	16
Common acute lymphoblastic leukemia	41	37
Acute lymphoblastic leukemia	1	1
<b>Total</b>	<b>269</b>	<b>230</b>
<b>Other leukemias</b>		
Acute myelomonocytic leukemia	15	2
Acute monocytic leukemia	4	1
Acute megakaryoblastic leukemia	6	0
<b>Total</b>	<b>25</b>	<b>3</b>
<b>T cell derived</b>		
Chronic lymphocytic leukemia (T cell)	11	0
Sézary syndrome/mycosis fungoides	6	0
Peripheral T cell lymphoma	25	0
Lymphoblastic lymphoma, thymic type	20	0
Lymphoblastic leukemia	12	0
<b>Total</b>	<b>74</b>	<b>0</b>
<b>Miscellaneous</b>		
Carcinoma	20	0
Fibrosarcoma	2	0
Osteosarcoma	1	0
<b>Total</b>	<b>23</b>	<b>0</b>

Staining was performed on cryostat tissue sections as described in the text, or by the APAAP immunoenzymatic procedure on blood or bone marrow smears, or on cytocentrifuge preparations. In all cases scored as positive, the majority (>70%) of neoplastic cells gave a positive reaction.

\*Centroblastic-centrocytic lymphoma is approximately equivalent to follicular lymphoma in other classifications.

†Centroblastic and immunoblastic lymphoma are equivalent to "histiocytic lymphoma, diffuse" in the Rappaport classification.

were immunoprecipitated is in keeping with a similar report from Moldenhauer and colleagues on two other anti-CD22 antibodies.<sup>19</sup>

The two anti-CD22 antibodies clearly delineated B cell follicles in cryostat sections of lymphoid tissue and did so with greater clarity than did either B1 (anti-CD20) or B2 (anti-CD21). Hofman and co-workers<sup>20</sup> also found that antibody B1 labeled germinal center cells but only a proportion of mantle zone lymphocytes; in contrast, Bhan and co-workers<sup>21</sup> reported labeling of all cells in lymphoid folli-



cles (both mantle zones and germinal centers). This discrepancy may relate to the relative sensitivities of labeling methods. When adjacent lymphoid tissue sections were stained with antibody B1 and anti-CD22 antibodies, however, the former reagent consistently gave weaker reactions. In this context, the initial detection of the two anti-CD22 antibodies by screening on tissue sections (rather than by binding to cell suspensions, used in screening for antibody B1) may account for their better reactivity as immunohistological reagents.

Anti-CD22 antibodies thus appear to be valuable reagents for labeling B cells in tissue sections and also in cell smears, and are comparable in our experience to antibodies against the 95,000 mol wt B cell-associated marker CD19 (first detected by antibody B4).<sup>22</sup> They appear to be specific for B cells, with the exceptions of weak reactions with FDRCs and epithelioid histiocytes. Furthermore, the reaction of anti-CD22 with epithelioid histiocytes and FDRCs was less pronounced than the labeling of these cells by antibodies B1 and B2, respectively.

As shown in Table 1, positive labeling with the anti-CD22 antibodies was obtained in most B cell neoplasms in tissue sections or cell smears. The negative reactions of cases of myeloma is in keeping with observations that a number of other human B cell antigens are lost during terminal differentiation to plasma cells.<sup>2</sup> The only positive reactions against neoplastic cells other than B cells were those occasionally observed with cells from myeloid leukemia (Table 1). This may be related to the observation that CD22 is weakly

expressed by epithelioid histiocytes (ie, by another cell type of myeloid/monocytic origin).

The broad spectrum of reactivity of anti-CD22 antibodies against neoplastic B cells observed in the present study contrasts with other reports that the CD22 antigen is undetectable on many B cell neoplasms.<sup>2</sup> This discrepancy may be related to reports that the CD22 antigen is initially found during B cell maturation in the cell cytoplasm, and only subsequently emerges onto the cell surface.<sup>19,23</sup> In consequence, blast cells in cases of common ALL contain cytoplasmic CD22 but lack this antigen on their surface.<sup>19,23</sup> Phorbol ester stimulation of common ALL and pre-B cell lines can induce membrane expression of CD22, however.<sup>23</sup> The practical implication of these observations is that immunocytochemical staining methods that allow antibodies to gain access to the cell interior (ie, in fixed cell smears or tissue sections) will reveal cytoplasmic CD22, whereas staining of cells in suspension will only reveal surface membrane antigen.

Whatever the explanation of the higher positivity rate for CD22 antigen on neoplastic B cells in the present study as compared with previous reports,<sup>2</sup> it is evident that these monoclonal anti-CD22 antibodies will detect most B cell neoplasms studied in tissue sections or cell smears. Because identification of neoplastic cell lineage on the basis of at least two independent markers is advisable, we believe that in routine diagnostic practice a combination of anti-CD19 and CD22 antibodies constitutes a reliable pair of reagents for detecting B cell neoplasms.

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# Expression of Human B Cell-Associated Antigens on Leukemias and Lymphomas: A Model of Human B Cell Differentiation

By Kenneth C. Anderson, Michael P. Bates, Bruce L. Slaughenhaupt, Geraldine S. Pinkus, Stuart F. Schlossman, and Lee M. Nadler

A series of monoclonal antibodies that define B cell restricted and associated antigens was utilized in an attempt to characterize tumors of B lineage and to relate these tumors to B cell differentiative stages. Antigens that were previously shown to be B cell restricted on normal B lymphocytes were similarly expressed only on B cell malignancies. In contrast, antigens that were B cell associated were also found on tumors of other lineages. Moreover, on the basis of cell surface phenotypes, tumors of B cell origin were divided into three major subgroups, which corre-

sponded to the level of differentiation of the malignant tumor cell: pre-B cell stage (non-T acute lymphoblastic leukemia and chronic myelocytic leukemia in lymphoid blast crisis); the mid-B cell stage (chronic lymphocytic leukemia, poorly differentiated lymphomas); and secretory B cell stage (large cell lymphomas and plasma cell tumors). A hypothetical model is derived that relates the malignant B cell to its normal cellular counterpart on the basis of cell surface expression of this panel of B cell-restricted and B cell-associated antigens.

**H**UMAN LEUKEMIAS and lymphomas have long been recognized as heterogeneous diseases by their morphological appearance, clinical presentation, and response to therapy.<sup>1-3</sup> Their classification has been traditionally based on cytologic appearance and cytochemical properties of the tumor<sup>4-9</sup> and, more recently, by immunologic markers that define both lineage and state of differentiation of the tumor cell.<sup>10-18</sup> Specifically, B cell tumors have been defined by their expression of cytoplasmic immunoglobulin (cIg)<sup>10</sup> or monoclonal surface immunoglobulin (sIg).<sup>11,12</sup> In an attempt to better characterize B cell tumors, several laboratories have developed monoclonal antibodies directed at antigens that are largely restricted to normal and malignant B cells and distinct from previously described B cell-associated markers, including Ia, sIg, Fc, and C3 receptors.<sup>19-29</sup> Nine such B cell-associated antigens have been reported from this laboratory: B1,<sup>20,30</sup> B2,<sup>22</sup> B4,<sup>27</sup> and PC-1,<sup>29</sup> which are B cell restricted within the hematopoietic system; Ia,<sup>31</sup> CALLA,<sup>32-35</sup> T1,<sup>36,37</sup> T10,<sup>38,39</sup> and PCA-1,<sup>28</sup> which are B cell associated as they are also expressed on other lymphoid and myeloid cells.

The B cell-specific antigens B1, B2, B4, and PC-1, which have previously been shown to be phenotypically and molecularly distinct from known B cell determinants, are expressed at limited stages of B cell differen-

tiation.<sup>20,22,27,29</sup> These antigens differ from one another in their expression on B cells: B1 and B4 span most of B cell differentiation, whereas B2 and PC-1 are expressed at limited stages of B cell differentiation, i.e., the midstage and plasma cell stage, respectively. A second group of B cell-associated markers, including Ia, CALLA, T1, T10, and PCA-1, in contrast to the former group, are not restricted to B lymphocytes within the hematopoietic system, but may appear on granulocytes, monocytes, T lymphocytes, and null cells.<sup>29,31-38</sup> Although the wider lineage distribution of these antigenic markers limits their utility when used alone, in combination with other markers they provide a very useful fingerprint of both the malignant and normal cell. The combination of both B cell restricted and associated antigens have clearly complemented conventional morphological and cytochemical techniques for the characterization of both normal B cell differentiation and malignant B cell categorization.<sup>40-43</sup>

In the studies to be described below, we have analyzed approximately 700 lymphomas and leukemias with a panel of monoclonal antibodies defining both B cell restricted and associated antigens. The analysis of large numbers of tumors with an extensive panel of antibodies has permitted the definition of their relationship to normal hematopoietic cells. Moreover, our studies suggest that B cell tumors fit into defined stages of normal B cell differentiation.

## MATERIALS AND METHODS

### *Production of Monoclonal Antibodies*

The techniques of immunization, somatic cell hybridization, and selection of hybridomas that were used to produce the monoclonal antibodies directed at the Ia, B4, CALLA, B1, B2, sIg, T1, T10, PC-1, and PCA-1 antigens have been previously described.<sup>43-46</sup>

### *Reactivity on Normal Tissues*

The reactivity and specificity of the monoclonal antibodies employed in this study on Ficoll-Hypaque mononuclear cells,<sup>47</sup> E+

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(T) cells, affinity-purified sIg positive<sup>48,49</sup> or immune rosette negative<sup>50</sup> (B) cells, adherent monocytes,<sup>51</sup> granulocytes,<sup>52</sup> and erythrocytes obtained both from peripheral blood and from lymphoid tissues, including tonsil, lymph node, spleen, bone marrow, and thymus, have been previously described in detail.<sup>20-22,27-40</sup> The expression of these antigens on B cells, as well as their cross-reactivity on cells of other lineages, is summarized in Table 1.

### Human Leukemia, Lymphoma, and Myeloma Samples

After appropriate human protection committee validation and informed consent, human samples were obtained for study. The 661 tumor samples were obtained from the Dana-Farber Cancer Institute, as well as from the Brigham and Women's, Beth Israel, and Massachusetts General Hospitals. Tumor cells obtained from patients with acute and chronic leukemia, non-Hodgkin's lymphomas, and plasma cell dyscrasias, in all instances, contained >75% neoplastic cells by Wright-Giemsa morphology. These tumors were classified histopathologically, utilizing a modified Rappaport system.<sup>5</sup> B lineage was defined by the presence of monoclonal sIg, as determined by indirect immunofluorescence with the use of anti- $\gamma$ ,  $\mu$ ,  $\lambda$ , and  $\kappa$  monoclonal antibodies.<sup>40</sup> T cell tumors were identified by reactivity with specific T cell monoclonal antibodies including anti-T3<sup>37,53,54</sup> and anti-T11.<sup>54,55</sup> Myeloid lineage was established by standard morphological and histochemical techniques, as well as by reactivity with the anti-MY7 and MY8<sup>56</sup> and anti-Mo1<sup>57</sup> monoclonal antibodies. All tissue specimens were immediately placed in medium containing 5% fetal calf serum (FCS), finely minced with forceps and scissors, and made into single-cell suspension by extrusion through stainless steel mesh. Tumor cell samples were either used fresh or cryopreserved in  $-196^{\circ}\text{C}$  vapor-phase liquid nitrogen in 10% dimethyl sulfoxide (DMSO) and 20% human serum until the time of surface characterization by indirect immunofluorescence.

### Indirect Immunofluorescence

Tumor cells were analyzed for surface phenotype by indirect immunofluorescence as previously described.<sup>58</sup> In brief,  $0.5-1 \times 10^6$  viable washed cells were treated with 100  $\mu\text{l}$  of a specific or control unreactive monoclonal antibody at saturating concentration, incubated at  $4^{\circ}\text{C}$  for 30 min, and washed 3 times. Cells were then treated

with 100  $\mu\text{l}$  of a 1:40 dilution of goat anti-mouse IgG and goat anti-mouse IgM conjugated with fluorescein isothiocyanate (FITC) (Coulter Electronics, Hialeah, FL) and incubated at  $4^{\circ}\text{C}$  for 30 min. After washing, tumor cells were analyzed for immunofluorescence using an Epics V cell sorter (Coulter), and the percent positive cells was determined using the EASY system immunoprogram (Coulter).

In addition to measuring the presence or absence of antigen expression on tumor cells, the relative intensity of antigen expression was also assayed qualitatively utilizing flow cytometry. This was done in an effort to define heterogeneity within the histologically defined subgroups of B cell tumors, as well as to relate the malignant cells to their normal cellular counterparts. Because antigen intensity, as assessed by indirect immunofluorescence, is dependent on both the number of antigenic determinants per cell and the cell surface area, we have attempted to compare cells of similar size within each of the histologically defined subgroups of B cell leukemias and lymphomas utilizing flow cytometric gating. The intensity of antigen expression on the tumor cell surface was qualitatively assessed utilizing the scale displayed in Fig. 1(A-D): weak immunofluorescent staining (+), moderate staining (++), strong immunofluorescence (+++), and brightest staining (++++) . Although heterogeneity of individual antigen intensity was observed within each phenotypic subgroup, a common phenotype based on the presence or absence of cell surface antigens as well as intensity of antigen expression was usually evident.

## RESULTS

The expression of a panel of B cell restricted and associated antigens on tumor cells isolated from patients with lymphoid and myeloid leukemias and non-Hodgkin's lymphomas is depicted in Table 2. A total of 661 patients were studied, consisting of 459 patients with leukemia, 165 patients with lymphoma, and 37 patients with plasma cell tumors. As shown in Table 2, the distribution of B4, B1, B2, sIg, and PC-1 antigens, known to be restricted, are limited in their expression to tumors of B cell origin, including non-T

Table 1. Reactivity of Monoclonal Antibodies

Monoclonal Antibody	B Cell Reactivity	Cross-Reactivity	Molecular Weight (kD)	References
Anti-Ia	All B cells, excluding plasma cell	Activated T cell, CFU-C, null cell (%), monocyte	29, 34	31
Anti-B4	All B cells following Ia <sup>+</sup> pre-B cell, excluding plasma cell	None	40, 80	27
Anti-CALLA	Pre-B cell	Granulocyte (weak)	100	21, 32-34
Anti-B1	All B cells following Ia <sup>+</sup> B4 <sup>+</sup> CAL <sup>-</sup> LA <sup>+</sup> pre-B cell, excluding plasma cell	None	35	20, 30
Anti-B2	B cells following Ia <sup>+</sup> B4 <sup>+</sup> B1 <sup>+</sup> cu <sup>+</sup> pre-B cell, lost with sIgD with appearance of presecretory IgM	None	140	22
Anti-sIg	All B cells following Ia <sup>+</sup> B4 <sup>+</sup> B1 <sup>+</sup> cu <sup>+</sup> pre-B cell, excluding plasma cell	None	IgG 150 IgM 900 IgD 150	39
Anti-T1	Subpopulation of B1 <sup>+</sup> B cells	Mature T cell	67	35, 36
Anti-T10	Activated B cell and plasma cell	Prothymocyte and thymocyte, activated T cell	45	37, 38
Anti-PCA-1	Plasma cell	Granulocyte, monocyte (weak)	24	28
Anti-PC-1	Plasma cell	None	28	29

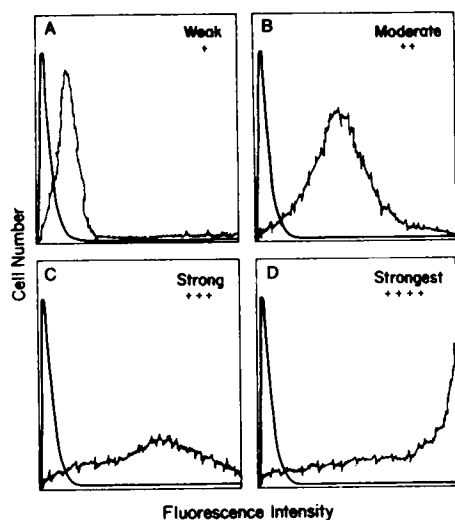


Fig. 1. The intensity of antigen expression on the tumor cell surface was qualitatively assessed as (A) weak (+), (B) moderate (\*\*), (C) strong (+++), and (D) strongest (++++). Background fluorescence staining (smooth line) was obtained by incubating cells with an isotype-identical unreactive monoclonal antibody and developing with G/M-FITC.

cell acute lymphoblastic leukemia (non-T ALL), lymphoid blast crisis of chronic myelocytic leukemia (CML-BC), B cell chronic lymphocytic leukemia (B-CLL), and B cell non-Hodgkin's lymphomas. In contrast, these antigens are not expressed on 37 patients with T cell tumors, including acute lympho-

blastic leukemia (T-ALL), chronic lymphocytic leukemia (T-CLL), and T lymphomas. Moreover, all samples from 154 patients with myeloid leukemias, including acute myeloblastic (AML) or myelomonoblastic leukemia (AMMoL) and chronic myelocytic leukemia in stable phase (CML), were negative.

The B4 antigen has the broadest expression of the B cell-restricted antigens, as the vast majority of B cell tumors are reactive with anti-B4. The B1 antigen displays a similar distribution to B4 except that those leukemias that appear earliest in ontogeny, i.e., non-T ALL and CML in lymphoid blast crisis, often lack B1. The B2 antigen, on the other hand, is expressed more narrowly on B cell malignancies, i.e., rarely on non-T ALL (5%), rarely if ever on lymphoid CML-BC (0%), on most B-CLL (90%), and on 68% of B cell lymphomas. Surface immunoglobulin (sIg), although expressed on over 80% of B cell CLL and lymphomas, is not expressed on either the earliest non-T ALL and lymphoid CML-BC or the most differentiated tumors. The PC-1 antigen, the most restricted of the B cell antigens, is wholly absent from B cell tumors, except for plasma cell tumors. These studies support the view that the B cell-restricted B4, B1, B2, sIg, and PC-1 antigens are expressed only on distinct subgroups of B cell tumors.

A panel of monoclonal antibodies directed against B cell-associated antigens was also examined for reactivity on this same group of tumor cells (Table 2). The

Table 2. Expression of B Lineage Antigens on Leukemias and Lymphomas

Leukemias	No. of Patients	Percentage of Patients Expressing Antigen									
		B Cell Restricted					B Cell Associated				
		B4	B1	B2	sIg	PC-1	Ia	CALLA	T1	T10	PCA-1
B cell											
Non-T ALL*	117	94	56	5††	0	0	100	88	ND	ND	0
CML-BC†	12	92	50	0	0	0	100	66	ND	ND	0
CLL‡	153	99	99	90	88	0	98	2	94	ND	0
T cell											
ALL	15	0	0	0	0	0	33	7	8	100	0
CLL	8	0	0	0	0	0	25	13	100	13	0
Myeloid											
AML/AMMOL§	148	0	0	0	0	0	100	0	ND	ND	3
CML	6	0	0	0	0	0	100	0	ND	ND	0
Lymphomas											
B cell¶	151	93	93	68	83	0	96	33	16	ND	0
T cell**	14	0	0	0	0	0	20	20	100	100	0
Plasma cell tumors††	37	6	6	0	0	100	16	0	ND	100	100

\*CALLA positive and CALLA negative non-T acute lymphoblastic leukemia.

†Chronic myelocytic leukemia in lymphoid blast crisis.

‡Chronic lymphocytic leukemia.

§Acute myeloblastic and acute myelomonoblastic leukemia.

|| Chronic myelocytic leukemia in stable phase.

¶B cell lymphomas include nodular and diffuse poorly differentiated (N and D-PDL), nodular mixed (NM), and large cell non-Hodgkin's lymphomas.

\*\*T cell lymphoblastic lymphoma.

††Plasma cell tumors include Waldenström's macroglobulinemia, myeloma, plasma cell leukemia (PCL), and plasmacytoma.

‡‡Two of 40 non-T ALL patients tested.

Ia-like antigen (Ia) is expressed on nearly all B cell leukemias and lymphomas, but, in addition, is also found on 25%–33% of T cell leukemias and lymphomas and the majority of myeloid leukemias. The CALLA antigen is expressed on most (88%) non-T ALL, but in contrast, is only expressed on a smaller subset (<10%) of other B cell leukemias and 33% of B cell lymphomas. Although not found on AML or AMMoL of myeloid derivation, the CALLA antigen is expressed on a fraction of T cell leukemias and lymphomas (10%–20%), as previously described.<sup>33</sup> The T1 antigen, known to be expressed on mature T lymphocytes and a fraction of thymocytes,<sup>36,37</sup> is also present in the overwhelming majority of patients with B-CLL (94%). This distribution is rather restricted, as only cells from a small fraction of B cell lymphomas (16%) are reactive. The T10 antigen is similarly present on malignancies of T lineage as well as on the B-lineage plasma cell neoplasms. Finally, the PCA-1 antigen, which is strongly expressed on plasma cell tumors, is rarely (3%) and only weakly expressed on tumor cells isolated from patients with myeloid leukemias. Although the latter group of antigens are not B-lineage restricted, they are nonetheless useful, as their expression at times is limited to distinct stages of neoplastic B cell differentiation.

Utilizing the panel of B cell restricted and associated antigens, it is possible to subdivide the non-T ALL into four subgroups. Of 117 patients with non-T ALL who were analyzed in the present study, 110 (94% patients) coexpressed Ia and B4 (Table 3) and

could therefore be subdivided into the following subgroups: 11 were Ia<sup>+</sup>B4<sup>+</sup>CALLA<sup>-</sup>B1<sup>-</sup>cyto-μ<sup>-</sup> (10% patients); 33 expressed Ia<sup>+</sup>B4<sup>+</sup>CALLA<sup>+</sup>B1<sup>-</sup>cyto-μ<sup>-</sup> (30% patients); 44 bore Ia<sup>+</sup>B4<sup>+</sup>CALLA<sup>+</sup>B1<sup>+</sup>cyto-μ<sup>-</sup> (40% patients); 22 had the Ia<sup>+</sup>B4<sup>+</sup>CALLA<sup>+</sup>B1<sup>+</sup>cyto-μ<sup>+</sup> cell surface phenotype (20% patients). The latter four groups defined virtually all of the non-T ALL studied. A few patients lacking both T cell and myeloid antigens and presumably non-T had unusual phenotypes, including Ia<sup>-</sup>B4<sup>-</sup>, Ia<sup>+</sup>B4<sup>-</sup>, and Ia<sup>-</sup>B4<sup>+</sup>. The latter patients accounted for <5% of the total population of non-T ALL analyzed. The pattern of cell surface antigens seen on lymphoid CML-BC tumor cells is similar to that described above for non-T ALL, suggesting that this tumor also corresponds to the earliest stages of malignant B cell ontogeny.

Examination of the antigen intensity on each of these subgroups suggests that these antigens appear sequentially in B cell ontogeny (Table 4). The Ia antigen is strongly expressed (Fig. 1C) on all of the early B cell tumors. The B4 antigen, although varying from moderate to strong in intensity (Fig. 1, B and C), is clearly positive on nearly all non-T ALL; moreover, it is expressed on non-T ALL to a greater degree than on any other B cell tumor. The CALLA antigen is moderately to strongly expressed on most non-T ALL, whereas the B1 antigen is weakly to moderately (Fig. 1, A and B) expressed on approximately 60% of these tumors. As noted in Table 3, only 2 of 40 non-T ALL studied with anti-B2 were B2 antigen positive. Whether this represents another "late" subgroup is

Table 3. Expression of B Lineage Antigens on B Cell Tumors

	No. of Patients	Percentage of Patients Expressing Antigen									
		Ia	B4	CALLA	B1	B2	slg	T1	T10	PCA-1	PC-1
Leukemias											
Non-T ALL *	117	100	94	88	56	5††	0	ND	ND	0	0
CML-BC†	12	100	92	66	50	0	0	ND	ND	0	0
CLL‡	153	98	99	2	99	90	88	94	ND	0	0
Lymphomas											
D-PDL§	52	100	94	15	92	67	94	19	ND	0	0
N-PDL	29	96	100	69	100	62	72	10	ND	0	0
NM¶	10	100	100	60	90	80	100	ND	ND	0	0
Large cell	60	90	87	10	90	18	77	ND	ND	0	0
Waldenstrom**	6	100	100	0	100	0	50	ND	100	100	100
Myeloma	22	0	0	0	0	0	0	ND	100	100	100
PCL††	6	0	0	0	0	0	0	ND	100	100	100
Plasmacytoma	3	0	0	0	0	0	0	ND	100	100	100

\*CALLA positive and CALLA negative non-T acute lymphoblastic leukemia.

†Chronic myelocytic leukemia in lymphoid blast crisis.

‡Chronic lymphocytic leukemia.

§Diffuse poorly differentiated lymphocytic lymphoma.

|| Nodular poorly differentiated lymphocytic lymphoma.

¶Nodular mixed lymphoma.

\*\*Waldenstrom's macroglobulinemia.

††Plasma cell leukemia.

‡‡Two of 40 non-T ALL patients tested.

Table 4. Intensity of Antigen Expression on B Cell Tumors

	No. of Patients	Cell Surface Antigen									
		Ia	B4	CALLA	B1	B2	sIg	T1	T10	PCA-1	PC-1
Leukemias											
Non-T ALL *	117	+++	++/+++	++/+++	0/+++	0	0	ND	ND	0	0
CML-BC†	12	+++	++/+++	++/+++	0/+++	0	0	ND	ND	0	0
B-CLL‡	153	++/+++	+/+	0	++	0/+	0/+	+/+	ND	0	0
Lymphomas											
D-PDL§	52	++/+++	+/+	0	++/+++	+/+	+/+	0/+	ND	0	0
N-PDL	29	++/+++	+/+	+/+	++/+++	+/+	+/+	0/+	ND	0	0
NM¶	10	++/+++	+/+	+/+	++/+++	+/+	+/+	ND	ND	0	0
Large cell	60	++/+++	+	0	++/+++	0	0/+	ND	ND	0	0
Waldenström**	6	+/+	0/+	0	0/+	0	0/+	ND	+	+	0/+
Myeloma	22	0	0	0	0	0	0	ND	+	++/+++	+++
PCL††	6	0	0	0	0	0	0	ND	+	++/+++	+++
Plasmacytoma	3	0	0	0	0	0	0	ND	0/+	++/+++	+++

\* CALLA positive and CALLA negative non-T acute lymphoblastic leukemia.

† Chronic myelocytic leukemia in blast crisis.

‡ B cell chronic lymphocytic leukemia.

§ Diffuse poorly differentiated lymphocytic lymphoma.

|| Nodular poorly differentiated lymphocytic lymphoma.

¶ Nodular mixed lymphoma.

\*\* Waldenström's macroglobulinemia.

†† Plasma cell leukemia.

still to be defined. Surface Ig, as well as the plasma cell antigens PC-1 and PCA-1, are absent on non-T ALL. Utilizing this panel of monoclonal reagents, one can therefore define heterogeneity within the histologic category of non-T ALL, i.e., Ia, B4, and CALLA have the broadest expression, B1 is expressed on fewer patients, and B2, sIg, PCA-1, and PC-1 are rarely if ever positive.

In addition to the above, there is an additional level of heterogeneity that relates to intensity of antigen expression within each phenotypic subgroup. For example, within the Ia<sup>+</sup>B4<sup>+</sup>CALLA<sup>+</sup>B1<sup>+</sup>cyto-μ<sup>-</sup> subgroup, the expression of B1 varies from barely detectable to strong. The precise meaning of this variability in antigen intensity within a histologically defined group is unclear.

The majority of B cell leukemias and lymphomas correspond to the midstages of malignant B cell differentiation, which are heralded by the acquisition and loss of new antigens, including B2 and the isotypes of sIg, and in some cases, T1 and CALLA. The coexpression of Ia, B4, B1, B2, and sIg defines the phenotype of most B-CLL (Table 3). Tumor cells from most patients with B-CLL express either very faint cell surface IgM or coexpress IgM and IgG and wholly lack the CALLA cell surface antigen. Of great interest is the observation that the majority of patients with B-CLL (94%) also express the T cell-associated antigen, T1. Although this represents the most common phenotype seen for B-CLL, it is clear that subgroups of B-CLL were defined that lack detectable sIg and/or B2. These observations again show that, within a morphologically defined group of patients with B-CLL, subgroups can be identified based on immunologic cell surface heterogeneity. The antigen intensity on B-CLL also distinguished these tumors from non-T

ALL and B lymphomas (Table 4). As was true for the non-T ALL, the Ia antigen can be moderately to strongly expressed on B-CLL; however, the B4 antigen is more weakly expressed on B-CLL than on non-T ALL. In contrast to non-T ALL, where the B1 antigen can be weak or absent, most B-CLL moderately express the B1 antigen. The CALLA antigen is lost and the B2 antigen appears to be acquired at the B-CLL stage. Both integral surface Ig and B2, however, are invariably only weakly expressed when present. The plasma cell restricted (PC-1) and associated (PCA-1) antigens are wholly absent on these B-CLL tumors.

B cell diffuse poorly differentiated lymphocytic lymphomas of both the nodular (NPDL) and diffuse (DPDL) subtypes also correspond to the midstages of malignant B cell differentiation (Table 3). Like the earlier B-CLL, the overwhelming majority of these tumors coexpress Ia, B4, B1, B2, and sIg. In contrast to B-CLL, the nodular PDL (69%) also express the CALLA antigen, and relatively few (10%) express the T1 antigen. CALLA, it should be noted, is rather unique in that it is expressed on the non-T ALL, disappears at the stage of B cell CLL, and reappears in a significant proportion of B cell lymphomas. Within PDL, the diffuse can be distinguished from the nodular subtypes by their lack of cell surface CALLA expression. The intensity of expression of these antigens on the cell surface can be of further differential value (Table 4). Although B-CLL and DPDL strongly express Ia and lack CALLA, PCA-1, and PC-1, they differ in that the expression of B4 and T1 is of lesser intensity and the expression of B1, B2, and sIg antigens of greater intensity on DPDL than on B-CLL. The nodular mixed (NM) lymphomas are phenotypically identical to NPDL, including the expression of cell

surface CALLA antigen. The moderate to strong expression of B2 on NPDL and NM lymphomas places these tumors at the mid-range of neoplastic B cell differentiation.

The terminal stage of B cell differentiation appears to begin with the transformed (B lymphoblast) B cell and proceeds to the final step of B cell differentiation, the plasma cell (Table 3). The large cell lymphomas coexpress Ia, B4, B1, and sIg, but lack CALLA and B2. Also absent are the plasma cell antigens PCA-1 and PC-1. Although the intensity of Ia and B1 expression remains strong, that of B4 and sIg is weaker on large cell lymphomas than on the above-mentioned NPDL, DPDL, and NM B cell tumors (Table 4). Waldenstrom cells demonstrate coexpression of Ia, B4, and B1, with only 50% of these cells expressing sIg and none B2 or CALLA cell surface antigens. The Waldenstrom cell is the first B lineage tumor to express either the plasma cell-associated antigens T10 and PCA-1 or the plasma cell-restricted antigen PC-1. The intensity of the Ia, B4, B1, and sIg antigens is less on the Waldenstrom cell than on large cell lymphomas. Nonetheless, the coexpression of the B cell (Ia, B4, B1, sIg) and plasma cell (T10, PCA-1, PC-1) antigens supports the notion that the Waldenstrom tumor cell is in "transition" from a malignant B cell to a malignant plasma cell. Lastly, all myelomas, whether obtained as tumor cells from the bone marrow, as plasma cell leukemias, or as solitary plasmacytomas, lack most B cell-restricted and B cell-associated antigens. These terminally differentiated B cells uniformly express T10, PCA-1, and PC-1. Both PCA-1 and PC-1 are moderately to strongly expressed on plasma cell tumors (Table 4) and represent the most discrete distribution of any of the antigens.

## DISCUSSION

In the present report, we have characterized approximately 700 leukemias and lymphomas with a panel of monoclonal antibodies that defines B cell-associated and B cell-restricted antigens in an attempt to define a broad overview of their distribution on normal tissues,

and more importantly, to relate B cell tumors to normal B cell differentiation. The present report is the first comparison of recently described antigens, i.e., B4, PCA-1, and PC-1, with other previously described antigens (B1, B2, etc.) on a large number of malignancies and allows an appreciation of B cell tumor heterogeneity that could not be obtained with either single reagents or small numbers of patients. Antigens that were previously shown to be restricted to normal B lymphocytes were similarly expressed only on B cell malignancies. In contrast, antigens that were B cell associated were also found on tumors of other lineages. The expression of these B cell restricted and associated antigens on B cell leukemias and lymphomas revealed distinct patterns of antigen expression within each group of histologically defined tumors. Tumor cells of B cell origin are capable of being divided into three major subgroups that correspond to the level of differentiation of the malignant tumor cell. These subgroups include the pre-B cell stage, the mid-B cell stage, and the secretory B cell stage. A hypothetical model relating the malignant B cell to its normal cellular counterpart on the basis of cell surface phenotype is depicted in Fig. 2.

The non-T ALL examined in this study appear to correspond to the pre-B cell stage of differentiation. Although morphologically identical, tumor cells from patients with non-T ALL could be subdivided into phenotypically defined subgroups on the basis of Ia, B4, CALLA, B1, and cyto- $\mu$ . The B cell origin of these tumors is now based on several lines of evidence. Initially, investigators demonstrated that tumor cells from approximately 15% of patients with non-T ALL contain  $\mu$ -chains without light chains and correspond phenotypically to pre-B cells.<sup>59</sup> Further studies demonstrated that approximately 50% of non-T ALL expressed the B1 antigen, suggesting that they were of B cell lineage.<sup>30</sup> The Ia<sup>+</sup>CALLA<sup>+</sup>B1<sup>-</sup> non-T ALL were next shown to be of B cell origin, as they could be induced by tumor promoters or conditioned media to express B1 and cyto- $\mu$ .<sup>60</sup> The more recent observation that almost all non-T ALL expressed the B cell-restricted B4 antigen provided additional evidence that

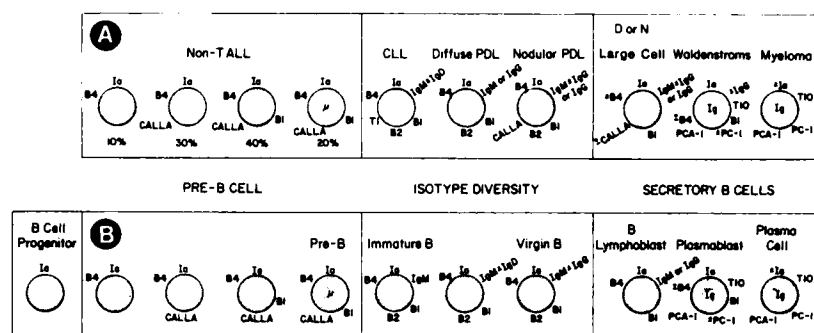


Fig. 2. Hypothetical model which relates the malignant B cell to its normal cellular counterpart on the basis of cell surface phenotype. (A) Human B cell tumors. (B) Normal B cell counterparts.



all of these tumors were of B cell origin.<sup>61</sup> Recent data demonstrating that all non-T ALL studied exhibited immunoglobulin gene rearrangements characteristic of B cells<sup>62,63</sup> provided additional evidence that virtually all non-T ALL were of B cell lineage.

The orderly acquisition of antigens on non-T ALL supports the view that these leukemic cells provide a model of normal pre-B cell differentiation. The observation that all non-T ALL strongly express the Ia antigen indicates that Ia develops very early on B cell precursors. B4 is most strongly expressed on the non-T cell ALL and CML in lymphoid blast crisis and decreases in intensity on more mature tumors. The CALLA antigen is expressed on slightly fewer non-T ALL than B4. It appears to follow B4 in B cell ontogeny. The B1 antigen follows the appearance of CALLA and is expressed on only 50% of non-T ALL. Hokland et al. have recently demonstrated that approximately half of CALLA<sup>+</sup> cells isolated from fetal lymphoid organs<sup>34</sup> and normal bone marrow<sup>35</sup> coexpress B1, and only 20% of these cells are cyto- $\mu$ <sup>+</sup>. This observation, combined with earlier studies demonstrating that CALLA<sup>+</sup>Ia<sup>+</sup> non-T ALL can be driven to express both B1 and cyto- $\mu$ , suggests that these are pre-B cells that precede the conventional cyto- $\mu$  pre-B cell stage in normal B cell ontogeny. The B1 antigen appears to be relatively weak on these tumor cells; it is strongly expressed only on the most mature non-T ALL, concomitant with the acquisition of cyto- $\mu$  chains. Thus, several B cell-restricted antigens appear prior to the conventional cyto- $\mu$ <sup>+</sup> pre-B cell and develop in a sequential fashion. The quantitation of antigen intensity for each of these markers may provide a model for their appearance in normal B cell ontogeny.

The tumors corresponding to the midstages of B cell differentiation are heralded by the appearance of new antigens and more mature morphology. The tumors included in these stages of B cell differentiation include B cell CLL and poorly differentiated lymphomas (Fig. 2). Multiple investigators have demonstrated the presence of T1 on B-CLL.<sup>64-73</sup> In our experience, B cell CLL phenotypically correspond to an immature B cell, which coexpresses Ia, B4, B1, B2, sIg, and T1.<sup>74</sup> Although normal B cells have not been thought to express the T1 antigen,<sup>64-73</sup> Caligaris-Cappio et al. have isolated cells that are surface immunoglobulin positive (sIg<sup>+</sup>) and mouse red blood cell receptor positive (MRBC<sup>+</sup>) from normal lymph node and tonsil, but not from peripheral blood.<sup>75</sup> In contrast, using dual fluorescent techniques, we can identify a small subset of circulating peripheral blood B cells and B cells isolated from tonsil and spleen that coexpress the B1 and T1 antigens.<sup>74</sup> Although these

cells coexpress sIg, B2, and B4, they differ from peripheral blood cells because they do not express the C3 B receptor. The B-CLL cell, therefore, may be the neoplastic counterpart of this small subpopulation of normal B lymphocytes and may not be reflective of the majority of circulating B cells. Moreover, although virtually all B-CLL also express Ia, B4, and B1, small numbers do lack sIg, B2, and T1. Whether this defines a degree of heterogeneity within this histologically comparable group is not clear. In addition, whereas CLL appears to be a relatively homogeneous clinical and morphological entity, significant heterogeneity of age at presentation, organ localization, and disease course is seen,<sup>76</sup> which may relate to its phenotypic diversity.

The diffuse and nodular PDL also correspond to stages within the mid-range of B cell differentiation (Fig. 2). These cells appear to correspond to the virgin B cell, as evidenced by their coexpression of Ia, B4, B1, B2, IgM, and IgG. In contrast to B-CLL cells, the intensity of immunoglobulin is considerably stronger, as is the intensity of B1 expression. The B2 and B4 antigens, although slightly less intense than on B-CLL cells, do not define significant heterogeneity. Nodular PDLs appear to uniformly express the CALLA antigen, whereas DPDL does not. These observations suggest that nodular and diffuse PDL may be derived from distinct populations of B cells. Moreover, previous experiments in our laboratory have shown that the CALLA antigen may be very weakly expressed on the germinal center cell within the secondary follicle of normal lymph node,<sup>77</sup> providing further evidence that nodular PDLs are of germinal center cell origin. It is important to note that there is clear-cut heterogeneity of antigen expression within the poorly differentiated lymphomas defined by the presence or absence of sIg and/or B2. For example, the majority of NPDL coexpress sIg and B2, but there are those subgroups that lack each of the antigens. These observations on tumors corresponding to the stages of mid-B cell differentiation complement the observations of Warnke and Link,<sup>78</sup> namely, that histologically defined tumors are in fact phenotypically heterogeneous. Moreover, the heterogeneity of antigen expression suggests that histologically comparable B cell tumors may be derived from both unique and distinct subpopulations of B lymphocytes. Clearly, an understanding of the function of many of these cell surface antigens will go far in examining some of the heterogeneity noted.

The third and final stage of B cell differentiation includes the secretory cells of B lineage. Tumors corresponding to this stage include diffuse and nodular large cell lymphomas, Waldenstrom's macroglobulin-

emias, and plasma cell tumors. The large cell lymphomas appear to correspond to the transformed B lymphoblast (Ia, B4, B1, sIg) (Fig. 2). These cells appear to express little or no B2 and large amounts of sIg and B1. This phenotypic pattern is similar to the "transformed" B lymphoblast obtained by treating normal B cells with pokeweed mitogen (PWM).<sup>27</sup> Waldenstrom's tumors, in contrast, coexpress B1 and B4, as well as the plasma cell-associated antigens PCA-1 and PC-1. The observed phenotype supports the notion that this cell represents a "transition" from the B lymphocyte to the lymphoplasmacytoid cell. The loss of B1, B2, and B4, coupled with the acquisition of PCA-1 and PC-1 on plasma cell dyscrasias, correlates with the *in vitro* pokeweed mitogen driven induction of normal B cell differentiation, in that loss of the B1 and B4 antigens occurs concomitant with the acquisition of cIg, surface T10, PCA-1, and PC-1 as well as plasmacytoid morphology.<sup>28,29</sup> This stage of B cell differentiation appears to be much more homogeneous within each of the histologically defined subgroups than has been seen at the early and mid-stages of B cell differentiation.

In the present study, we have utilized single cell suspensions isolated from patients with leukemias and lymphomas in an attempt to phenotypically correlate the malignant B cell with its normal B cell differentiative counterpart. In contrast to the immunoperoxidase technique, which preserves tissue architecture but does not permit accurate assessment of either antigen inten-

sity or localization on the cell surface, flow cytometry of single cell suspensions of tumor cells allows one to both determine the cell surface phenotype and assess antigen intensity. One pitfall of indirect immunofluorescence and flow cytometric analysis is that small amounts of antigen may be present on individual tumor cell surfaces that are not detected by this technique. In addition, the antigen intensity on the tumor cell surface depends on both the number of antigenic determinants per cell and the size of the tumor cell, requiring one to either compare tumor cells of identical size or to calculate mean fluorescence density. In the present report, considering the large number of patients studied, we have attempted to assess antigen density on tumor cells of similar size by selected cell size gating on the fluorescence activated cell sorter. Attempts have also been made to examine cells visually with a fluorescent microscope in order to confirm our flow cytometric observations on cell antigen density. We clearly believe that the examination of tumor cells in single suspension and in tissue section by immunologic techniques will complement one another and existing techniques. New insight into diagnoses and biologic behavior of these B cell leukemias and lymphomas is likely to occur through a combination of approaches. The utilization of cell surface markers as defined above should permit us to not only define these tumors as cells of B lineage, but also to accurately identify their state of differentiation and their relation to normal B cell subpopulations.

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